11-13 Immunological memory is long-lived after infection or vaccination.

Most children in developed countries are now vaccinated against measles virus; before vaccination was widespread, most were naturally exposed to this virus and developed an acute, unpleasant, and potentially dangerous illness. Whether through vaccination or infection, children exposed to the virus acquire long-term protection from measles, lasting for most people for the whole of their life. The same is true of many other acute infectious diseases (see Chapter 16): this state of protection is a consequence of immunological memory.

The basis of immunological memory has been hard to explore experimentally. Although the phenomenon was first recorded by the ancient Greeks and has been exploited routinely in vaccination programs for more than 200 years, it is only now becoming clear that this memory reflects a small population of specialized **memory cells** formed during the adaptive immune response that can persist in the absence of the antigen that originally induced them. This mechanism of maintaining memory is consistent with the finding that only individuals who were themselves previously exposed to a given infectious agent are immune, and that memory is not dependent on repeated exposure to infection as a result of contacts with other infected individuals. This was established by observations made of populations on remote islands, where a virus such as measles can cause an epidemic, infecting all people living on the island at that time, after which the virus disappears for many years. On reintroduction from outside the island, the virus does not affect the original population but causes disease in those people born since the first epidemic.

Studies have attempted to determine the duration of immunological memory by evaluating responses in people who received vaccinia, the virus used to immunize against smallpox (Fig. 11.17). Because smallpox was eradicated in 1978, it is presumed that their responses represent true immunological memory and are not due to restimulation from time to time by the smallpox virus. The study found strong vaccinia-specific CD4 and CD8 T-cell memory responses as long as 75 years after the original immunization, and from the strength of these responses it was estimated that the memory response had an approximate half-life of between 8 and 15 years. Half-life represents the time that a response takes to reduce to 50% of its original strength. Titers of antivirus antibody remained stable, without measurable decline.

These findings show that immunological memory need not be maintained by repeated exposure to infectious virus. Instead, it is likely that memory is sustained by long-lived antigen-specific lymphocytes that were induced by the original exposure and that persist until a second encounter with the pathogen. Although most of the memory cells are in a resting state, careful studies have shown that a small percentage of these cells are dividing at any one time. What stimulates this infrequent cell division is unclear, but it is likely that cytokines produced either constitutively or during antigen-specific immune responses directed at other, non-cross-reactive, antigens are responsible. The number of memory cells for a given antigen is highly regulated, remaining practically constant during the memory phase, which reflects a control mechanism that maintains a balance between cell proliferation and cell death.

Immunological memory can be measured experimentally in various ways. Adoptive transfer assays (see Appendix I, Section A-36) of lymphocytes from animals immunized with simple, nonliving antigens have been favored for such studies, because the antigen cannot proliferate. In these experiments, the existence of memory cells is measured purely in terms of the transfer of specific responsiveness from an immunized, or 'primed,' animal to a nonimmunized recipient, as tested by a subsequent immunization with the antigen. Animals that received memory cells have a faster and more robust response to

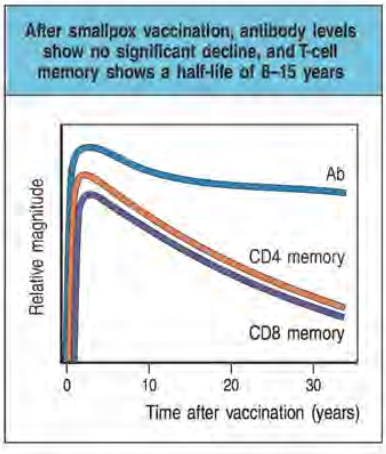


Fig. 11.17 Antiviral immunity after smallpox vaccination is long lived. Because smallpox has been eradicated, recall responses measured in people who were vaccinated for smallpox can be taken to represent true memory in the absence of reinfection. After smallpox vaccination, antibody levels show an early peak with a period of rapid decay, which is followed by long-term maintenance that shows no significant decay. CD4 and CD8 T-cell memory is long-lived but gradually decays, with a half-life in the range 8–15 years.

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antigen challenge than do controls that did not receive cells, or that received cells from nonimmune donors.

Experiments like these have shown that when an animal is first immunized with a protein antigen, functional helper T-cell memory against that antigen appears abruptly and reaches a maximum after 5 days or so. Functional antigen-specific B-cell memory appears some days later, then enters a phase of proliferation and selection in lymphoid tissue. By 1 month after immunization, memory B cells are present at their maximum level. These levels of memory cells are then maintained, with little alteration, for the lifetime of the animal. It is important to recognize that the functional memory elicited in these experiments can be due to the precursors of memory cells as well as the memory cells themselves. These precursors are probably activated T cells and B cells, some of whose progeny will later differentiate into memory cells. Thus, precursors to memory can appear very shortly after immunization, even though resting memory-type lymphocytes may not yet have developed.

In the following sections we look in more detail at the changes that occur in lymphocytes after antigen priming that lead to the development of resting memory lymphocytes, and discuss the mechanisms that might account for these changes.

11-14 Memory B-cell responses differ in several ways from those of naive B cells.

Immunological memory in B cells can be examined quite conveniently *in vitro* by isolating B cells from immunized or unimmunized mice and restimulating them with antigen in the presence of helper T cells specific for the same antigen (Fig. 11.18). B cells from immunized mice produce responses that differ both quantitatively and qualitatively compared with naive B cells from unimmunized mice. B cells that respond to the antigen increase in frequency by up to 100-fold after their initial priming in the primary immune response, and, as a result of the process of affinity maturation (see Section 10-8), produce antibody of higher affinity than unprimed B lymphocytes. The response observed from immunized mice is due to **memory B cells**, which we introduced in Section 11-9 as B cells that arise from the germinal center reaction. These cells populate the spleen and lymph nodes as well as circulating through the blood, and express some markers that distinguish them from naive B cells and plasma cells. In the human, a marker of memory B cells is CD27, a member of the TNF receptor family that is also expressed by naive T cells and binds the TNF-family ligand CD70, which is expressed by dendritic cells (see Section 9-13).

	Source of B cells	
	Unimmunized donor Primary response	Immunized donor Secondary response
Frequency of antigen-specific B cells	$1:10^4 - 1:10^5$	$1:10^2 - 1:10^3$
Isotype of antibody produced	IgM > IgG	IgG, IgA
Affinity of antibody	Low	High
Somatic hypermutation	Low	High

Fig. 11.18 The generation of secondary antibody responses from memory B cells is distinct from the generation of the primary antibody response. These responses can be studied and compared by isolating B cells from immunized and unimmunized donor mice, and stimulating them in culture in the presence of antigen-specific effector T cells. The primary response usually consists of antibody molecules made by plasma cells derived from a quite diverse population of precursor B cells specific for different epitopes of the antigen and with receptors with a range of affinities for the antigen. The antibodies are of relatively low affinity overall, with few somatic mutations. The secondary response derives from a far more limited population of high-affinity B cells, which have, however, undergone significant clonal expansion. Their receptors and antibodies are of high affinity for the antigen and show extensive somatic mutation. The overall effect is that although there is usually only a 10–100-fold increase in the frequency of activatable B cells after priming, the quality of the antibody response is radically altered, in that these precursors induce a far more intense and effective response.

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A primary antibody response is characterized by the initial rapid production of IgM, accompanied by an IgG response, due to class switching, which lags slightly behind it (Fig. 11.19). The secondary antibody response is characterized in its first few days by the production of relatively small amounts of IgM antibody and much larger amounts of IgG antibody, with some IgA and IgE. At the beginning of the secondary response, the source of these antibodies is memory B cells that were generated in the primary response and that have already switched from IgM to another isotypes, and express either IgG, IgA, or IgE on their surface, as well as a somewhat higher level of MHC class II molecules and B7.1 than is typical of naive B cells. The average affinity of IgG antibodies increases throughout the primary response and continues to increase during the ongoing secondary and subsequent responses (see Fig. 11.19). The higher affinity of memory B cells for antigen and their higher levels of cell-surface MHC class II molecules facilitate antigen uptake and presentation, which together with an increased expression of co-stimulatory molecules allows memory B cells to initiate their crucial interactions with helper T cells at lower doses of antigen than do naive B cells. This means that B-cell differentiation and antibody production start earlier after antigen stimulation than in the primary response. The secondary response is characterized by a more vigorous and earlier generation of plasma cells than in the primary response, thus accounting for the almost immediate abundant production of IgG (see Fig. 11.19).

The antibodies made in primary and secondary responses can be clearly distinguished in cases in which the primary response is dominated by antibodies that are closely related to each other and show little somatic hypermutation. This occurs in some inbred mouse strains in which certain haptens are recognized by a limited set of naive B cells. For example, in C57BL/6 mice, antibodies against the hapten nitrophenol (NP) are encoded by the same V_H (VH186.2) and V_L (λ1) genes in all animals of the strain. As a result of this uniformity in the primary response, changes in the antibody molecules produced in secondary responses to the same antigens are easy to observe. These differences include not only numerous somatic hypermutations in antibodies containing the dominant V regions (see Fig. 5.24) but also the addition of antibodies containing V_H and V_L gene segments not detected in the primary response. These are thought to derive from B cells that were activated at low frequency during the primary response, and thus were not detected, and which differentiated into memory B cells.

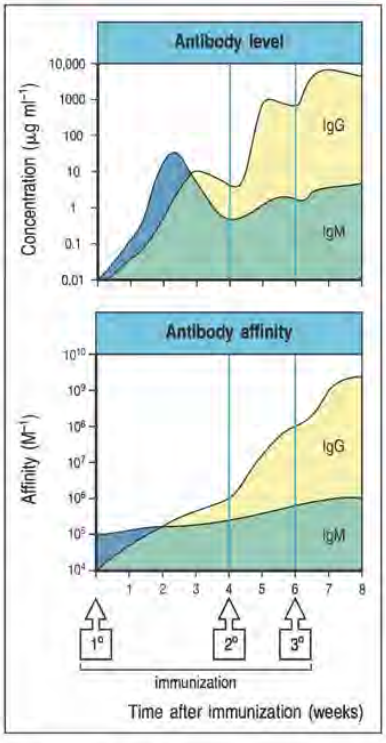


Fig. 11.19 Both the affinity and the amount of antibody increase with repeated immunization. The upper panel shows the increase in antibody concentration with time after a primary (1°), followed by a secondary (2°) and a tertiary (3°), immunization; the lower panel shows the increase in the affinity of the antibodies (affinity maturation). Affinity maturation is seen largely in IgG antibody (as well as in IgA and IgE, which are not shown) coming from mature B cells that have undergone isotype switching and somatic hypermutation to yield higher-affinity antibodies. The blue shading represents IgM on its own; the yellow shading IgG, and the green shading the presence of both IgG and IgM. Although some affinity maturation occurs in the primary antibody response, most arises in later responses to repeated antigen injections. Note that these graphs are on a logarithmic scale; it would otherwise be impossible to represent the overall increase of around a million-fold in the concentration of specific IgG antibody from its initial level.

11-15 Repeated immunization leads to increasing affinity of antibody due to somatic hypermutation and selection by antigen in germinal centers.

In secondary and subsequent immune responses, any antibodies persisting from previous responses are immediately available to bind the newly introduced pathogen. These antibodies divert the antigen to phagocytes for degradation and disposal (see Section 10-22), and if there is sufficient antibody to clear or inactivate the pathogen completely, it is possible that no secondary immune response will ensue. If the levels of pathogen overwhelm the amount of circulating antibody, excess antigens will bind to receptors on B cells and initiate a secondary B-cell response in the peripheral lymphoid organs. B cells with the highest avidity for antigen are the first to be recruited into this secondary response, and so memory B cells, which have already been selected for their avidity to antigen, make up a substantial part of the cells that contribute to the secondary response.

Secondary B-cell responses begin with the proliferation of B cells and T cells at the interface between the T-cell and B-cell zones, as in primary responses. Memory T cells reside in lymphoid tissues, but can also enter nonlymphoid tissues (see Section 11-6). Memory B cells, in contrast, continue to recirculate

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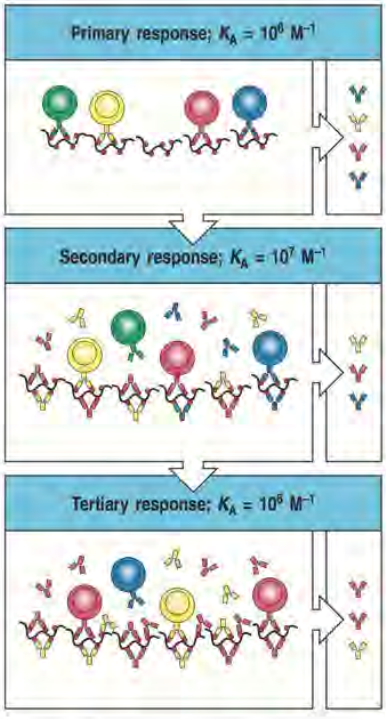


Fig. 11.20 The mechanism of affinity maturation in an antibody response. At the beginning of a primary response, B cells with receptors of a wide variety of affinities (K_A), most of which will bind antigen with low affinity, take up antigen, present it to helper T cells, and become activated to produce antibody of varying and relatively low affinity (top panel). These antibodies then bind and clear antigen, so that only those B cells with receptors of the highest affinity can continue to capture antigen and interact effectively with helper T cells. Such B cells will therefore be selected to undergo further expansion and clonal differentiation, and the antibodies they produce will dominate a secondary response (middle panel). These higher-affinity antibodies will in turn compete for antigen and select for the activation of B cells bearing receptors of still higher affinity in the tertiary response (bottom panel).

through the same secondary lymphoid compartments as naive B cells, principally the follicles of the spleen, lymph nodes, and the Peyer's patches of the gut mucosa. Memory B cells that have picked up antigen are able to present peptide:MHC class II complexes to their cognate helper T cells surrounding the follicle. Contact between the antigen-presenting B cells and helper T cells leads to the rapid proliferation of both the B cells and T cells. As the higher-affinity memory B cells compete most effectively for antigen, these B cells are most efficiently stimulated in the secondary immune response. Reactivated B cells that have not yet undergone differentiation into plasma cells migrate into the follicle and become germinal center B cells. There, they enter a second round of proliferation, during which the DNA encoding their immunoglobulin V domains undergoes somatic hypermutation, before differentiating into antibody-secreting plasma cells (see Section 10-8). The affinity of the antibodies produced rises progressively and rapidly, because B cells with the highest-affinity antigen receptors produced by somatic hypermutation bind antigen most efficiently, and will be selected to proliferate by their interactions with antigen-specific helper T cells in the germinal center (Fig. 11.20).

Memory B cells may not produce all antibodies in the secondary response. On secondary exposure to antigen, preexisting antibody can sometimes permit the formation of immune complexes, which do not form immediately in the primary response. Recent studies have shown that such immune complexes can bind to and activate signaling by Fc receptors on antigen-specific naive B cells, which acts to accelerate their kinetics of response. The formation of immune complexes, however, requires equivalent levels of antigen and antibody, which may not always occur, for example if antibody is present in great excess. Still, the shorter lag phase in antibody production in the secondary response may arise not only from the faster intrinsic responses of memory B cells, but also from accelerated naive B-cell responses if antigen:antibody complexes can form.

11-16 Memory T cells are increased in frequency compared with naive T cells specific for the same antigen, and have distinct activation requirements and cell-surface proteins that distinguish them from effector T cells.

Because the T-cell receptor does not undergo class switching or somatic hypermutation, it is not as easy to identify a memory T cell unequivocally as it is to identify a memory B cell. After immunization, the number of T cells reactive to a given antigen increases markedly as effector T cells are produced, and then falls back to persist at a level 100–1000-fold above the initial frequency for the rest of the life of the animal or person (Fig. 11.21). These persisting cells are designated **memory T cells**. They are long-lived cells with a particular set of cell-surface proteins, responses to stimuli, and expression of genes that control cell survival. Overall, their cell-surface proteins are similar to those of effector cells, but there are some distinctive differences (Fig. 11.22). In B cells, there is an obvious distinction between effector and memory cells, because effector B cells are terminally differentiated plasma cells that have already been activated to secrete antibody until they die.

A major problem in experiments aimed at establishing the existence of memory T cells is that many assays for T-cell effector function take several days, during which the putative memory T cells are reinduced to effector cell status. Thus, assays requiring several days do not distinguish preexisting effector cells from memory T cells, because memory cells can acquire effector activity during the period of the assay. This problem does not apply to cytotoxic T cells, however, because cytotoxic effector T cells can program a target cell for lysis in 5 minutes, whereas memory CD8 T cells need more time

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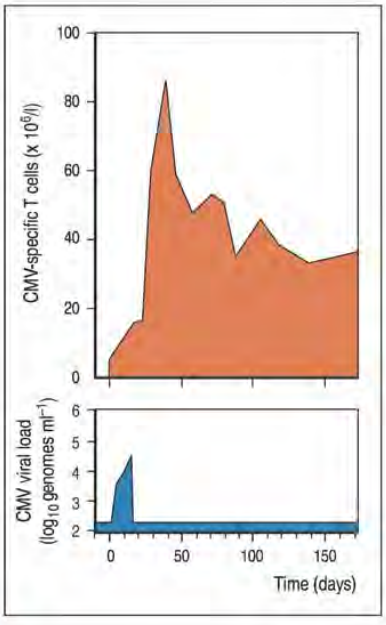


Fig. 11.21 Generation of memory T cells after a virus infection. After an infection, in this case a reactivation of latent cytomegalovirus (CMV), the number of T cells specific for viral antigen increases dramatically and then falls back to give a sustained low level of memory T cells. The upper panel shows the numbers of T cells (orange); the lower panel shows the course of the virus infection (blue), as estimated by the amount of viral DNA in the blood. Data courtesy of G. Aubert.

than this to be reactivated to become cytotoxic. Thus, their cytotoxic actions will appear later than those of any preexisting effector cells, even though they can become activated without undergoing DNA synthesis, as shown by studies conducted in the presence of mitotic inhibitors.

Recently it has become possible to track particular clones of antigen-specific CD8 T cells by staining them with tetrameric peptide:MHC complexes (see Appendix I, Section A-28). It has been found that the number of antigen-specific CD8 T cells increases markedly during an infection, and then decreases by up to 100-fold; nevertheless, this final level is distinctly higher than before priming. These cells continue to express some markers characteristic of activated cells, such as CD44, but stop expressing other activation markers, such

Protein	Naive	Effector	Memory	Comments
CD44	+	+++	+++	Cell-adhesion molecule
CD45RO	+	+++	+++	Modulates T-cell receptor signaling
CD45RA	+++	+	+++	Modulates T-cell receptor signaling
CD62L	+++	–	Some +++	Receptor for homing to lymph node
CCR7	+++	+/–	Some +++	Chemokine receptor for homing to lymph node
CD69	–	+++	–	Early activation antigen
Bcl-2	++	+/–	+++	Promotes cell survival
Interferon-γ	–	+++	+++	Effector cytokine; mRNA present and protein made on activation
Granzyme B	–	+++	+/–	Effector molecule in cell killing
FasL	–	+++	+	Effector molecule in cell killing
CD122	+/–	++	++	Part of receptor for IL-15 and IL-2
CD25	–	++	–	Part of receptor for IL-2
CD127	++	–	+++	Part of receptor for IL-7
Ly6C	+	+++	+++	GPI-linked protein
CXCR4	+	+	++	Receptor for chemokine CXCL12; controls tissue migration
CCR5	+/–	++	Some +++	Receptor for chemokines CCL3 and CCL4; tissue migration

Fig. 11.22 Expression of many proteins alters when naive T cells become memory T cells. Proteins that are differently expressed in naive T cells, effector T cells, and memory T cells include adhesion molecules, which govern interactions with antigen-presenting cells and endothelial cells; chemokine receptors, which affect migration to lymphoid tissues and sites of inflammation; proteins and receptors that promote the survival of memory cells; and proteins that are involved in effector functions, such as granzyme B. Some changes also increase the sensitivity of the memory T cell to antigen stimulation. Many of the changes that occur in memory T cells are also seen in effector cells, but some, such as expression of the cell-surface proteins CD25 and CD69, are specific to effector T cells; others, such as expression of the survival factor Bcl-2, are limited to long-lived memory T cells. This list represents a general picture that applies to both CD4 and CD8 T cells in mice and humans, but some details that may differ between these sets of cells have been omitted for simplicity.

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as CD69. In addition they express more Bcl-2, a protein that promotes cell survival and may be responsible for the long half-life of memory CD8 cells.

The α subunit of the IL-7 receptor (IL-7Rα or CD127) may be a good marker for activated T cells that will become long-lived memory cells (see Fig. 11.22). Naive T cells express IL-7Rα, but it is rapidly lost upon activation and is not expressed by most effector T cells. For example, during the peak of the effector response against lymphocytic choriomeningitis virus (LCMV) in mice, around day 7 of the infection, a small population of approximately 5% of CD8 effector T cells expressed high levels of IL-7Rα. Adoptive transfer of these cells, but not the effector T cells expressing low levels of IL-7Rα, could provide functional CD8 T-cell memory to uninfected mice (Fig. 11.23). This experiment suggests that the early maintenance, or the reexpression, of IL-7Rα identifies effector CD8 T cells that generate memory T cells, although it is still not known whether, and how, this process is regulated. Memory T cells are more sensitive to restimulation by antigen than are naive T cells, and more quickly and more vigorously produce several cytokines such as IFN-γ, TNF-α, and IL-2 in response to such stimulation. A similar progression occurs for T cells in humans after immunization with a vaccine against yellow fever virus.

CD4 T-cell memory has been more difficult to study, in part because their responses are smaller than those of CD8 T cells and also because, until recently, there were no peptide:MHC class II reagents similar to the peptide:MHC class I tetramers. Nevertheless, the transfer and priming of naive T cells carrying T-cell receptor transgenes that give the T cells a known peptide:MHC specificity has made it possible to visualize memory CD4 T cells. They appear as a long-lived population of cells that share some surface characteristics of activated effector T cells but are distinct from effector T cells in that they require additional restimulation before acting on target cells. Changes in three cell-surface proteins—L-selectin, CD44, and CD45—that occur on the putative memory CD4 T cells after exposure to antigen are particularly significant. L-selectin is lost on most memory CD4 T cells, whereas CD44 levels are increased on all memory T cells; these changes contribute to directing the migration of memory T cells from the blood into the tissues rather than directly into lymphoid tissue. The isoform of CD45 changes because of alternative splicing of exons that encode the extracellular domain of CD45, leading to isoforms, such as CD45RO, that are smaller and more readily associated with the T-cell receptor and facilitate antigen recognition (see Fig. 11.22). These changes are characteristic of cells that have been activated to become effector T cells, yet some of the cells on which these changes have occurred have many characteristics of resting CD4 T cells, suggesting that they represent memory CD4 T cells. Only after reexposure to antigen on an antigen-presenting cell do they achieve effector T-cell status and acquire all the characteristics of T_H2 or T_H1 cells, secreting IL-4 and IL-5, or IFN-γ, respectively.

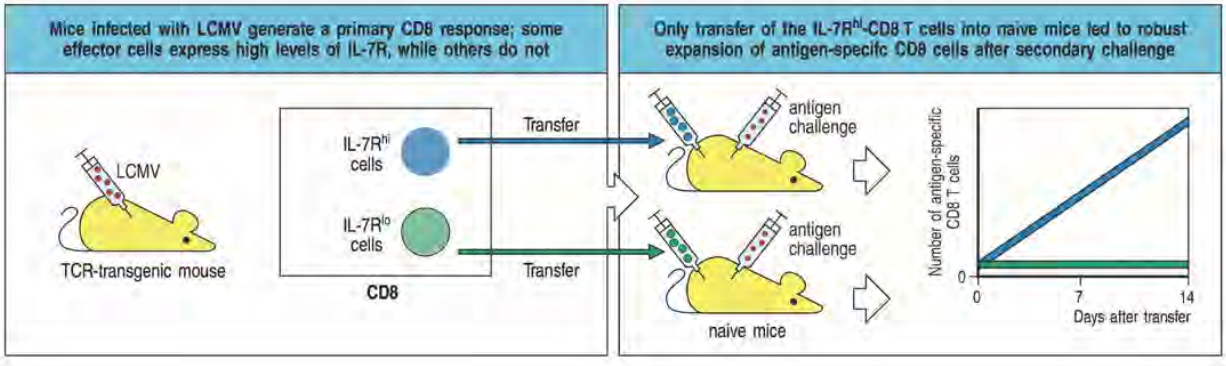


Fig. 11.23 Expression of the IL-7 receptor (IL-7R) indicates which CD8 effector T cells can generate robust memory responses. Mice expressing a T-cell receptor (TCR) transgene specific for a viral antigen from lymphocytic choriomeningitis virus (LCMV) were infected with the virus and effector cells were collected on day 11. Effector CD8 T cells expressing high levels of IL-7R (IL-7R^{hi}, blue) were separated and transferred into one group of naive mice, and effector CD8 T cells expressing low IL-7R (IL7R^{lo}, green) were transferred into another group. Three weeks after transfer, the mice were challenged with a bacterium engineered to express the original viral antigen, and the numbers of responding transferred T cells (detected by their expression of the transgenic TCR) were measured at various times after challenge. Only the transferred IL-7R^{hi} effector cells could generate a robust expansion of CD8 T cells after the secondary challenge.

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It therefore seems reasonable to designate these cells as memory CD4 T cells and to surmise that naive CD4 T cells differentiate into effector T cells, some of which then become memory cells. As with memory CD8 T cells, direct staining of CD4 T cells with peptide:MHC class II tetramers can identify antigen-specific CD4 T cells, and analysis using intracellular cytokine staining (see Appendix I, Section A-27) can determine whether they are T_H1, T_H17, or T_H2 cells. These improvements in the identification and phenotyping of CD4 T cells will rapidly increase our knowledge in this area and could contribute valuable comparative information on naive, memory, and effector CD4 T cells.

The homeostatic mechanisms governing the survival of memory T cells differ from those for naive T cells. Memory T cells divide more frequently than naive T cells, and their expansion is controlled by a balance between proliferation and cell death. As with naive cells, the survival of memory T cells requires stimulation by the cytokines IL-7 and IL-15. IL-7 is required for the survival of both CD4 and CD8 memory T cells, but in addition, IL-15 is critical for the long-term survival and proliferation of CD8 memory T cells under normal conditions. For memory CD4 T cells, the role of IL-15 is still under investigation.

In addition to cytokine stimulation, naive T cells also require contact with self-peptide:self-MHC complexes for their long-term survival in the periphery (see Section 8-29), but it seems that memory T cells do not have this requirement. It has been found, however, that memory T cells surviving after transfer to MHC-deficient hosts have some defects in typical T-cell memory functions, indicating that stimulation by self-peptide:self-MHC complexes may be required for their continued proliferation and optimal function (Fig. 11.24).

11-17 Memory T cells are heterogeneous and include central memory and effector memory subsets.

CD4 and CD8 T cells can differentiate into two types of memory cells with distinct activation characteristics (Fig. 11.25). One type is called an **effector memory cell** because it can rapidly mature into an effector T cell and secrete large amounts of IFN-γ, IL-4, and IL-5 early after restimulation. These cells lack the chemokine receptor CCR7 but express high levels of β_1 and β_2 integrins, as well as receptors for inflammatory chemokines. This profile suggests that these effector memory cells are specialized for rapidly entering inflamed tissues. The other type is called a **central memory cell**. It expresses CCR7 and would therefore be expected to recirculate more easily to the T-cell zones of peripheral lymphoid tissues, as do naive T cells. Central memory cells are very sensitive to cross-linking of their T-cell receptors and rapidly express CD40 ligand in response; however, they take longer than effector memory cells to differentiate into effector T cells and thus do not secrete such large amounts of cytokines early after restimulation.

The distinction between central memory cells and effector memory cells has been made both in humans and in the mouse. However, this general

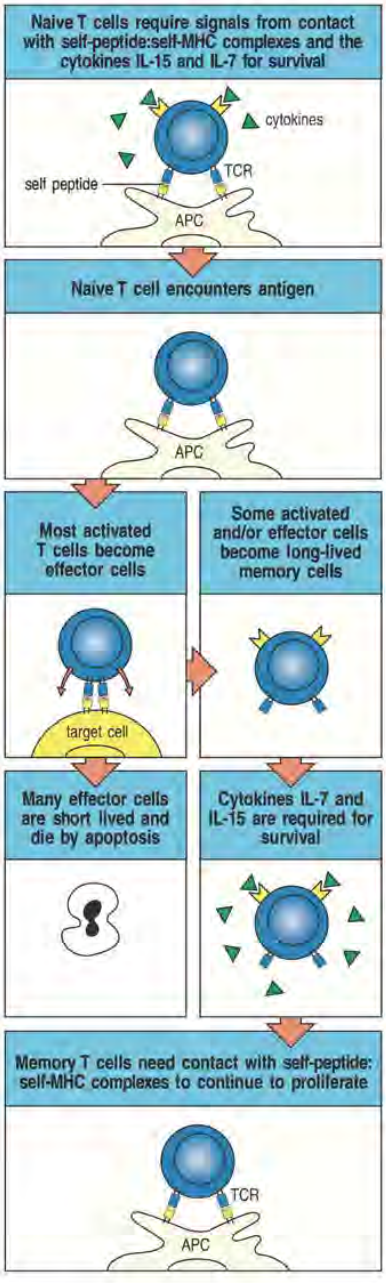


Fig. 11.24 Naive T cells and memory T cells have different requirements for survival. For their survival in the periphery, naive T cells require periodic stimulation with the cytokines IL-7 and IL-15 and with self antigens presented by MHC molecules. On priming with its specific antigen, a naive T cell divides and differentiates. Most of the progeny differentiate into relatively short-lived effector cells, but some effector cells become long-lived memory T cells, which need to be sustained by cytokines but do not require contact with self-peptide:self-MHC complexes purely for survival. However, contact with self antigens does seem necessary for memory T cells to continue to proliferate and thus keep up their numbers in the memory pool.

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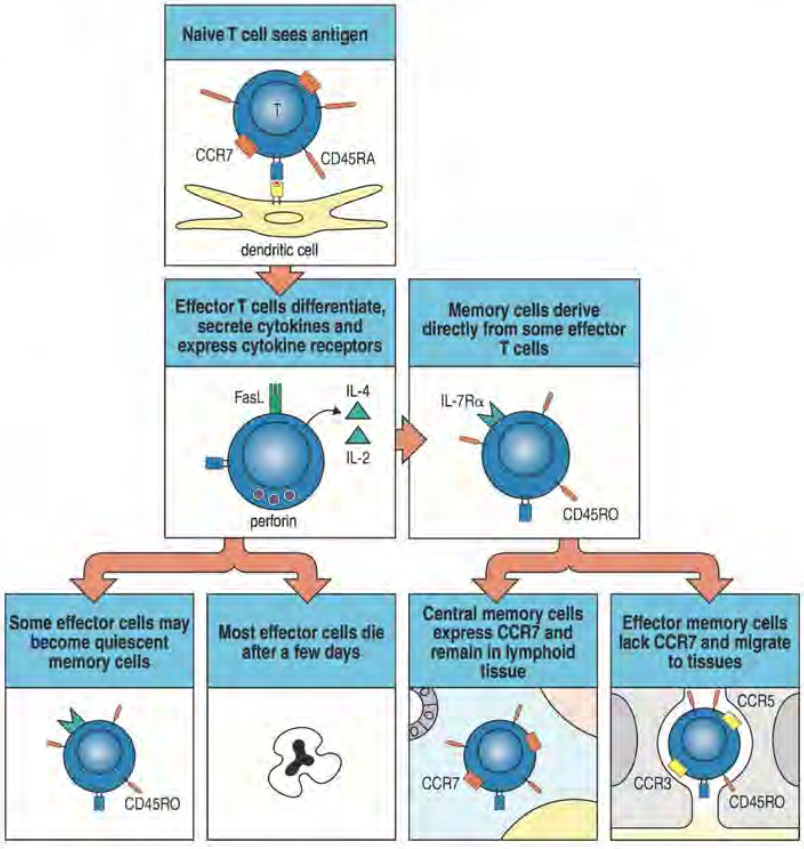


Fig. 11.25 T cells differentiate into central memory and effector memory subsets distinguished by expression of the chemokine receptor CCR7. Quiescent memory cells bearing the characteristic CD45RO surface protein can arise from activated effector cells (right half of diagram) or directly from activated naive T cells (left half of diagram). Two types of quiescent memory T cells can derive from the primary T-cell response. Central memory cells express CCR7 and remain in peripheral lymphoid tissues after restimulation. Memory cells of the other type—effector memory cells—mature rapidly into effector T cells after restimulation, and secrete large amounts of IFN-γ, IL-4, and IL-5. They do not express the receptor CCR7, but express receptors (CCR3 and CCR5) for inflammatory chemokines.

distinction does not imply that each subset is a uniform population. Within the CCR7-expressing central memory subset there are extensive differences in the expression of other markers, particularly receptors for other chemokines. For example, a subset of the CCR7-positive central memory cells also expresses CXCR5, similarly to T_{FH} cells, although it is not yet clear whether these memory cells can provide help to B cells in the germinal center.

On stimulation by antigen, central memory cells rapidly lose expression of CCR7 and differentiate into effector memory cells. Effector memory cells are also heterogeneous in the chemokine receptors they express, and have been classified according to chemokine receptors typical of T_H1 cells, such as CCR5, and of T_H2 cells, such as CCR4. Central memory cells are not yet committed to particular effector lineages, and even effector memory cells are not fully committed to the T_H1 or the T_H2 lineage, although there is some correlation between their eventual output of T_H1 or T_H2 cells and the chemokine receptors expressed. Further stimulation with antigen seems to drive the differentiation of effector memory cells gradually into the distinct effector T-cell lineages.

11-18 CD4 T-cell help is required for CD8 T-cell memory and involves CD40 and IL-2 signaling.

In Section 11-8 we saw how primary CD8 T-cell responses to *Listeria monocytogenes* can occur in mice that lack CD4 T cells. After 7 days of infection, wild-type mice and mice lacking CD4 T cells both showed equivalent expansion and activity of pathogen-specific CD8 effector T cells. However, they are not equally able to generate memory CD8 T cells. Mice that lack CD4 T cells as

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the result of a deficiency in MHC class II were found to generate much weaker secondary responses, characterized by many fewer expanding memory CD8 T cells specific for the pathogen. In this experiment, the *Listeria* carried a gene for the protein ovalbumin, and it was the response to this protein that was measured as a marker for CD8 T-cell memory (Fig. 11.26). CD4 T cells in these mice are lacking both during the primary response and in any secondary challenge, so the requirement for CD4 T cells could be either in the initial programming of CD8 T cells during their primary activation to enable memory development, or, alternatively, in providing help only during the secondary memory response.

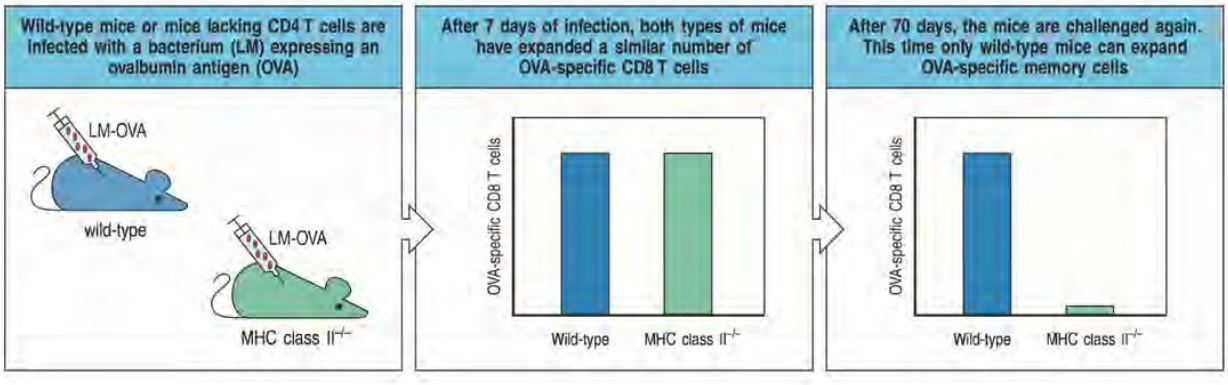
Other experiments indicate that this CD4 T-cell help is necessary for programming naive CD8 T cells to be able to generate memory cells capable of robust secondary expansion. Memory CD8 T cells that developed in the absence of CD4 help were transferred into wild-type mice. After transfer, the recipient mice were challenged again. In mice that received CD8 memory T cells that developed without CD4 T-cell help, the CD8 T cells showed a reduced ability to proliferate even though the recipient mice expressed MHC class II. This result indicates that CD4 T-cell help is required during the priming of CD8 T cells and not simply at the time of secondary responses. This requirement for CD4 help in CD8 memory generation has also been demonstrated by experiments in which CD4 T cells were depleted by treatment with antibody or in which mice were deficient in the CD4 gene.

The mechanism underlying this requirement for CD4 T cells is not completely understood. It may involve two types of signals received by the CD8 T cell—those received through CD40 and those received through the IL-2 receptor. CD8 T cells that do not express CD40 are unable to generate memory T cells. Although many cells could potentially express the CD40 ligand needed to stimulate CD40, it is most likely that CD4 T cells are the source of this signal.

The requirement for IL-2 signaling in programming CD8 memory was discovered by using CD8 T cells with a genetic deficiency in the IL-2Rα subunit, which were therefore unable to respond to IL-2. Because IL-2Rα signaling is required for the development of T_{reg} cells, mice lacking IL-2Rα develop a lymphoproliferative disorder. However, this disorder does not develop in mice that are mixed bone marrow chimeras harboring both wild-type and IL-2Rα-deficient cells, and these chimeras can be used to study the behavior of IL-2Rα-deficient cells. When these chimeric mice were infected with LCMV and their responses were tested, memory CD8 responses were found to be defective specifically in the T cells lacking IL-2Rα.

CD4 T cells also provide help in maintaining the numbers of CD8 memory T cells that is distinct from their effect in programming naive CD8 T cells to become memory cells (Fig. 11.27). When CD8 memory T cells are transferred

Fig. 11.26 CD4 T cells are required for the development of functional CD8 memory T cells. Mice that do not express MHC class II molecules (MHC $II^{-/-}$) fail to develop CD4 T cells. Wild-type and MHC $II^{-/-}$ mice were infected with *Listeria monocytogenes* expressing the model antigen ovalbumin (LM-OVA). After 7 days, the numbers of OVA-specific CD8 T cells can be measured by using specific MHC tetramers that contain an OVA peptide, and therefore bind to T-cell receptors that react with this antigen. After 7 days of infection, mice lacking CD4 T cells have the same number of OVA-specific CD8 T cells as wild-type mice do. However, when mice are allowed to recover for 60 days, during which time memory T cells develop, and are then rechallenged with LM-OVA, the mice lacking CD4 T cells fail to expand CD8 memory cells specific to OVA, whereas there is a strong CD8 memory response in the wild-type mice.



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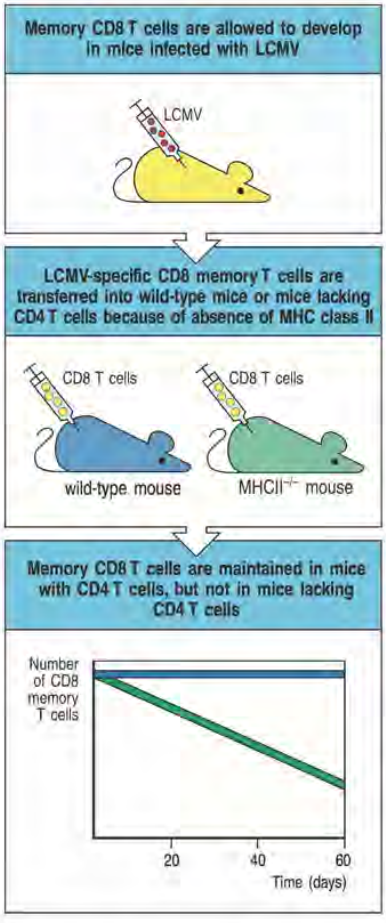


Fig. 11.27 CD4 T cells promote the maintenance of CD8 memory cells. The dependence of memory CD8 T cells on CD4 T cells is shown by the different lifetimes after transfer into host mice that either have normal CD4 T cells (wild-type), or lack CD4 T cells (MHC II$^{-/-}$). In the absence of MHC class II proteins, CD4 T cells fail to develop in the thymus. When CD8 memory T cells specific for LCMV were isolated from donor mice 35 days after infection with the virus and transferred into these hosts, memory cells were maintained only in mice that had CD4 T cells. The basis for this action of CD4 T cells is not yet clear, but has implications for conditions such as HIV-AIDS in which numbers of CD4 T cells are diminished.

into immunologically naive mice, the presence or absence of CD4 T cells in the recipient influences the maintenance of the CD8 memory cells. Transfer of CD8 memory cells into mice lacking CD4 T cells is followed by a gradual decrease in the number of memory cells in comparison with a similar transfer into wild-type mice. In addition, CD8 effector cells transferred into mice lacking CD4 T cells had a relative impairment of CD8 effector functions. These experiments show that the CD4 T cells activated during an immune response have a significant impact on the quantity and quality of the CD8 T-cell response, even when they are not needed for the initial CD8 T-cell activation. CD4 T cells help to program naive CD8 T cells to be able to generate memory T cells, help to promote efficient effector activity, and help to maintain memory T-cell numbers.

11-19 In immune individuals, secondary and subsequent responses are mainly attributable to memory lymphocytes.

In the normal course of an infection, a pathogen proliferates to a level sufficient to elicit an adaptive immune response and then stimulates the production of antibodies and effector T cells that eliminate the pathogen from the body. Most of the effector T cells then die, and antibody levels gradually decline, because the antigens that elicited the response are no longer present at the level needed to sustain it. We can think of this as feedback inhibition of the response. Memory T and B cells remain, however, and maintain a heightened ability to mount a response to a recurrence of infection with the same pathogen.

Antibody and memory lymphocytes remaining in an immunized individual can have the effect of reducing the activation of naive B and T cells on a subsequent encounter with the same antigen. In fact, passively transferring antibody to a naive recipient can be used to inhibit naive B cell responses to that same antigen. This phenomenon has been put to practical use to prevent Rh$^-$ mothers from making an immune response to a Rh$^+$ fetus, which can result in hemolytic disease of the newborn (see Appendix I, Section A-11). If anti-Rh antibody is given to the mother before she is first exposed to her child's Rh$^+$ red blood cells, her response will be inhibited. The mechanism of this suppression is likely to involve the antibody-mediated clearance and destruction of fetal red blood cells that have entered the mother, thus preventing naive B cells and T cells from mounting an immune response. Presumably, anti-Rh antibody is in excess over antigen, so that not only is antigen eliminated, but immune complexes are not formed to stimulate naive B cells through Fc receptors. Memory B-cell responses are, however, not inhibited by antibody, so the Rh$^-$ mothers at risk must be identified and treated before a primary response has occurred. Because of their high affinity for antigen and alterations in their B-cell receptor signaling requirements, memory B cells are much more sensitive to the small amounts of antigen that cannot be efficiently cleared by the passive anti-Rh antibody. The ability of memory B cells to be activated to produce antibody, even when exposed to preexisting antibody, also allows secondary antibody responses to occur in individuals who are already immune.

These suppressive mechanisms might also explain the phenomenon known as **original antigenic sin**. This term was coined to describe the tendency of people to make antibodies only against epitopes expressed on the first influenza virus variant to which they are exposed, even in subsequent infections with variants that bear additional, highly immunogenic, epitopes (Fig. 11.28). Antibodies against the original virus will tend to suppress responses of naive B cells specific for the new epitopes. This might benefit the host by using only those B cells that can respond most rapidly and effectively to the virus. This pattern is broken only if the person is exposed to an influenza virus that lacks

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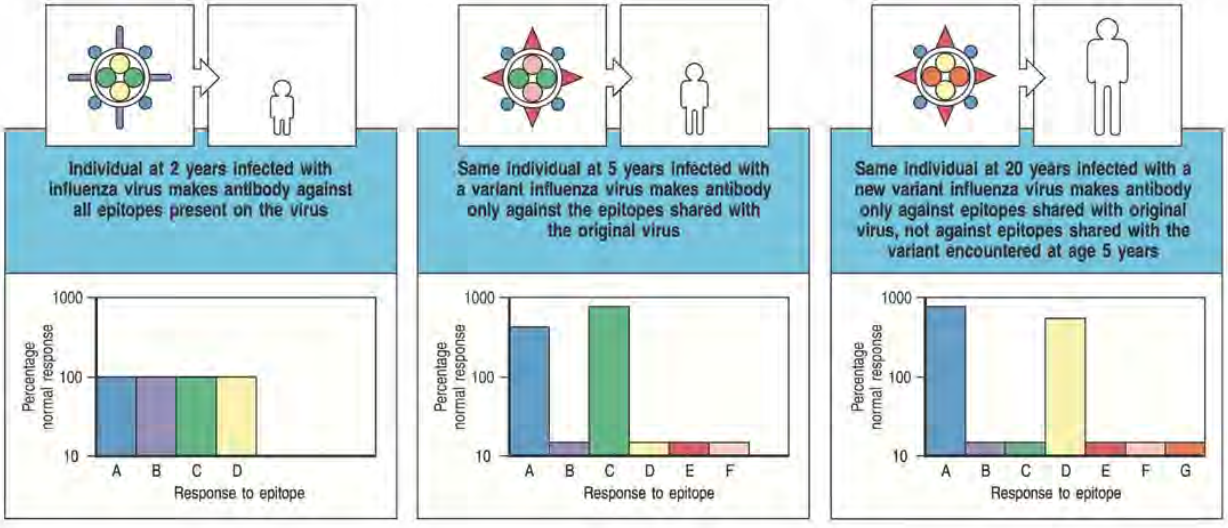


Fig. 11.28 When individuals who have been infected with one variant of influenza virus are infected with a second or third variant, they make antibodies only against epitopes that were present on the initial virus. A child infected for the first time with an influenza virus at 2 years of age makes a response to all epitopes (left panel). At age 5 years, the same child exposed to a different influenza virus responds preferentially to those epitopes shared with the original virus, and makes a smaller than normal response to new epitopes on the virus (center panel). Even at age 20 years, this commitment to respond to epitopes shared with the original virus, and the subnormal response to new epitopes, is retained (right panel). This phenomenon is called 'original antigenic sin.'

all epitopes seen in the original infection, because now no preexisting antibodies bind the virus, and naive B cells are able to respond.

A similar suppressive effect of antigen-specific memory T cells on naive T-cell responses has been observed in some settings, such as infection by lymphocytic choriomeningitis virus (LCMV) in the mouse or dengue virus in humans. For example, mice that were primed with one strain of LCMV responded to a subsequent infection with a variant LCMV by using CD8 T cells directed against antigens specific for the first, rather than the new, variant. However, this effect was not observed when responses to variable ovalbumin antigenic epitopes were examined in the setting of recurrent infections using the bacterial pathogen *Listeria monocytogenes*, suggesting that original antigenic sin may not be a universal occurrence in all immune responses.

Summary.

Protective immunity against reinfection is one of the most important consequences of adaptive immunity. Protective immunity depends not only on preformed antibody and effector T cells but most importantly on the establishment of a population of lymphocytes that mediate long-lived immunological memory. The capacity of memory cells to respond rapidly to restimulation with the same antigen can be transferred to naive recipients by primed B and T cells. The precise changes that distinguish naive, effector, and memory lymphocytes include the regulation of expression of receptors for cytokines, such as IL-7 and IL-15, that help to maintain these cells, and the regulation of chemokine receptors, such as CCR7, that distinguish between functional subsets of memory cells. The advent of receptor-specific reagents—MHC tetramers—has allowed an analysis of the relative contributions of clonal expansion and differentiation to the memory phenotype. Memory B cells can be distinguished by changes in their immunoglobulin genes because of isotype switching and somatic hypermutation, and secondary and subsequent immune responses are characterized by antibodies with increasing affinity for the antigen. As for T-cell memory, there is a complex interplay between CD4 and CD8 T cells that is only partly understood. Although CD8 T cells can generate effective primary responses in the absence of help from CD4 T cells, it is becoming clear that CD4 T cells have an integral role in regulating CD8 T-cell memory. These issues will be critical in understanding, for example, how to design effective vaccines for diseases such as HIV/AIDS.

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Summary to Chapter 11.

Vertebrates resist infection by pathogenic microorganisms in several ways. The innate defenses can act immediately and may succeed in repelling the infection, but if not they are followed by a series of induced early responses that help to contain the infection as adaptive immunity develops. These first two phases of the immune response rely on recognizing the presence of infection by using the nonclonotypic receptors of the innate immune system. They are summarized in Fig. 11.29 and covered in detail in Chapter 3. Specialized subsets of lymphocytes, which can be viewed as intermediates between innate and adaptive immunity, include iNKT cells, which can help to bias the CD4 T-cell response toward a T_H1 or a T_H2 phenotype, and NK cells, which can be recruited to lymph nodes and secrete IFN-γ, and thus promote a T_H1 response. The third phase of an immune response is the adaptive immune response (see Fig. 11.29), which is mounted in the peripheral lymphoid tissue that serves the particular site of infection and takes several days to develop, because T and B lymphocytes must encounter their specific antigen, proliferate, and differentiate into effector cells. T-cell dependent B-cell responses cannot be initiated until antigen-specific T_{FH} cells have had a chance to proliferate and differentiate. Once an adaptive immune response has occurred, the antibodies and effector T cells are dispersed via the circulation and recruited into the infected tissues; the infection is usually controlled and the pathogen is contained or eliminated. The final effector mechanisms used to clear an infection depend on the type of infectious agent, and in most cases they are the same as those employed in the early phases of immune defense; only the recognition mechanism changes and is more selective (see Fig. 11.29).

An effective adaptive immune response leads to a state of protective immunity. This state consists of the presence of effector cells and molecules produced in the initial response, and of immunological memory. Immunological memory

	Phases of the immune response		
	Immediate (0–4 hours)	**Early (4–96 hours)**	**Late (96–100 hours)**
	Nonspecific Innate No memory No specific T cells	Nonspecific + specific Inducible No memory No specific T cells	Specific Inducible Memory Specific T cells
Barrier functions	Skin, epithelia, mucins, acid	Local inflammation (C5a) Local TNF-α	IgA antibody in luminal spaces IgE antibody on mast cells Local inflammation
Response to extracellular pathogens	Phagocytes Alternative and MBL complement pathway Lysozyme Lactoferrin Peroxidase Defensins	Mannan-binding lectin C-reactive protein T-independent B-cell antibody Complement	IgG antibody and Fc receptor-bearing cells IgG, IgM antibody + classical complement pathway
Response to intracellular bacteria	Macrophages	Activated NK-dependent macrophage activation IL-1, IL-6, TNF-α, IL-12	T-cell activation of macrophages by IFN-γ
Response to virus-infected cells	Natural killer (NK) cells	IFN-α and IFN-β IL-12-activated NK cells	Cytotoxic T cells IFN-γ

Fig. 11.29 The components of the three phases of the immune response against different classes of microorganisms. The mechanisms of innate immunity that operate in the first two phases of the immune response are described in Chapters 2 and 3, and thymus-independent (T-independent) B-cell responses are covered in Chapter 10. The early phases contribute to the initiation of adaptive immunity, and they influence the functional character of the antigen-specific effector T cells and antibodies that appear on the scene in the late phase of the response. There are striking similarities in the effector mechanisms at each phase of the response; the main change is in the recognition structure used.

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is manifested as a heightened ability to respond to pathogens that have previously been encountered and successfully eliminated. Memory T and B lymphocytes have the property of being able to transfer immune memory to naive recipients. The mechanisms that maintain immunological memory include certain cytokines, such as IL-7 and IL-15, as well as homeostatic interactions between the T cell receptors on memory cells with self-peptide:self-MHC complexes. The artificial induction of protective immunity, which includes immunological memory, by vaccination is the most outstanding accomplishment of immunology in the field of medicine. The understanding of how this is accomplished is now catching up with its practical success. However, as we will see in Chapter 13, many pathogens do not induce protective immunity that completely eliminates the pathogen, so we will need to learn what prevents this before we can prepare effective vaccines against these pathogens.

Questions.

11.1 Communication is critical in any large enterprise. (a) How is the body alerted to an invasion by microbes, and (b) how does it ensure that its responses reach the site of infection?

11.2 The immune system responds to particular classes of pathogens in different ways. What properties of viruses and bacteria are used to induce T_H1 responses to them, and what host cells provide the information about the type of pathogen present?

11.3 Differentiated T cells require continued signals to maintain their function. (a) What signals do T_H1 cells need? (b) What advantages might the requirement for continued signals have? What disadvantages?

11.4 One could question the need for immunological memory. Invertebrates get by without adaptive immunity or memory. After all, if you survive the first infection without memory, you should be able to survive it the second time without memory. And if you fail to survive the first infection, memory is of no help. (a) What are the advantages of immunological memory that counter this argument? What features of pathogens might have driven the evolution of immunological memory? (b) Innate immune responses seem to lack memory, but may be augmented for a time after infection. What features of immunological memory provided by adaptive immunity are of greater value than simply an augmented innate response? In what way could these features be a disadvantage? Give an example.

11.5 Memory responses differ from primary immune responses in several important properties. Name three ways in which they differ, and describe the underlying mechanism(s) involved in each case.

11.6 (a) Discuss the relative roles of cytokine signals and signals received through the T-cell receptor in the survival and function of memory T cells. (b) Compare and contrast their requirements and responses to such signals with those of naive T cells.

11.7 You are swimming in freshwater and are infected by a parasite that entered your body through your skin. Outline how your body would generate an immune response against this pathogen. Would the response be T_H1 or T_H2 polarized? Include all relevant cell types and molecules that would be involved.

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12 The Mucosal Immune System

A series of anatomically distinct compartments can be distinguished within the immune system, each of which is specially adapted to generate a response to antigens encountered in a particular set of tissues. In previous chapters we have mainly discussed adaptive immune responses that are initiated in lymph nodes and spleen—the peripheral lymphoid tissues that respond to antigens that have entered the body via the skin, are present in the internal organs, or have spread into the blood. These are the immune responses most studied by immunologists, as they are the responses evoked when antigens are administered by injection. There is, however, an additional compartment of the adaptive immune system, of even greater size, located near the surfaces where most pathogens actually invade. This is the **mucosal immune system**—the subject of this chapter.

The organization of the mucosal system.

The thin layer of mucosal epithelium lining internal body surfaces is the only physical barrier against invasion of the underlying tissues by potential pathogens and the body's own commensal microorganisms, which are present in vast numbers at most mucosal surfaces. These surfaces therefore require continual protection against invaders. The epithelium can be breached relatively easily and so its barrier function needs to be supplemented by defenses provided by the cells and molecules of the mucosal immune system. The innate defenses of mucosal tissues, such as antimicrobial peptides and cells bearing invariant pathogen-recognition receptors, are described in Chapters 2 and 3. In this chapter we concentrate on the adaptive mucosal immune system. Many of the anatomical and immunological principles underlying the mucosal immune system apply to all its constituent tissues; here we will use the intestine as our example.

12-1 The mucosal immune system protects the internal surfaces of the body.

The mucosal immune system comprises the body surfaces lined by mucus-secreting epithelium—the gastrointestinal tract, the upper and lower respiratory tract, and the urogenital tract. It also includes the exocrine glands associated with these organs, such as the conjunctivae and lachrymal glands of the eye, the salivary glands, and the lactating breast (Fig. 12.1). The mucosal surfaces represent an enormous area to be protected. The human small intestine, for instance, has a surface area of almost 400 m^2, which is 200 times

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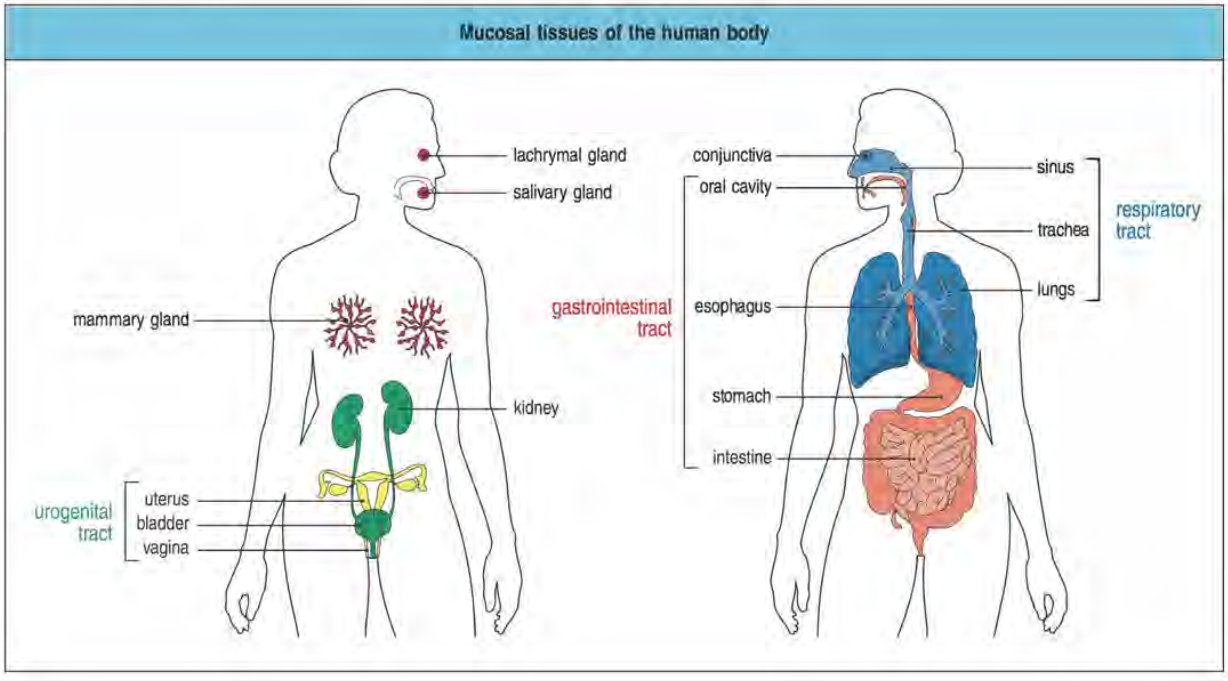


Fig. 12.1 The mucosal immune system. The tissues of the mucosal immune system are the lymphoid organs associated with the intestine, respiratory tract, and urogenital tract, as well as the oral cavity and pharynx and the glands associated with these tissues, such as the salivary glands and lachrymal glands. The lactating breast is also part of the mucosal immune system.

that of the skin. Because of their physiological functions in gas exchange (the lungs), food absorption (the gut), sensory activities (eyes, nose, mouth, and throat), and reproduction (uterus and vagina), the mucosal surfaces are thin and permeable barriers to the interior of the body. The importance of these tissues to life means that effective defense mechanisms are essential to protect them from invasion. Equally, their fragility and permeability create obvious vulnerability to infection, and it is not surprising that the vast majority of infectious agents invade the human body by these routes (Fig. 12.2). Diarrheal diseases, acute respiratory infections, pulmonary tuberculosis, measles, whooping cough, and worm infestations continue to be the major causes of death throughout the world, especially in infants in developing countries. To these must be added the human immunodeficiency virus (HIV), a pathogen whose natural route of entry via a mucosal surface is often overlooked, as well as other sexually transmitted infections such as syphilis.

The mucosal surfaces are also portals of entry for a vast array of foreign antigens that are not pathogenic. This is best seen in the gut, which is exposed to enormous quantities of food proteins—an estimated 30–35 kg per year per person. At the same time, the healthy large intestine is colonized by at least a thousand species of bacteria that live in symbiosis with their host and are known as **commensal microorganisms**, or the **microbiota**. These bacteria are present at levels of at least 10^{12} organisms per milliliter in the colon contents, making them the most numerous cells in the body by a factor of 10. In normal circumstances they do no harm and are beneficial to their host in many ways. Many of the other mucosal surfaces have equivalent, if smaller, populations of resident commensal organisms (Fig. 12.3).

As food proteins and the microbiota contain many foreign antigens, they are capable of being recognized by the adaptive immune system. Generating protective immune responses against these harmless agents would, however, be inappropriate and wasteful. Indeed, aberrant immune responses of this kind are now believed to be the cause of some relatively common diseases, including celiac disease (caused by a response to the wheat protein gluten) and inflammatory bowel diseases such as Crohn's disease (a response

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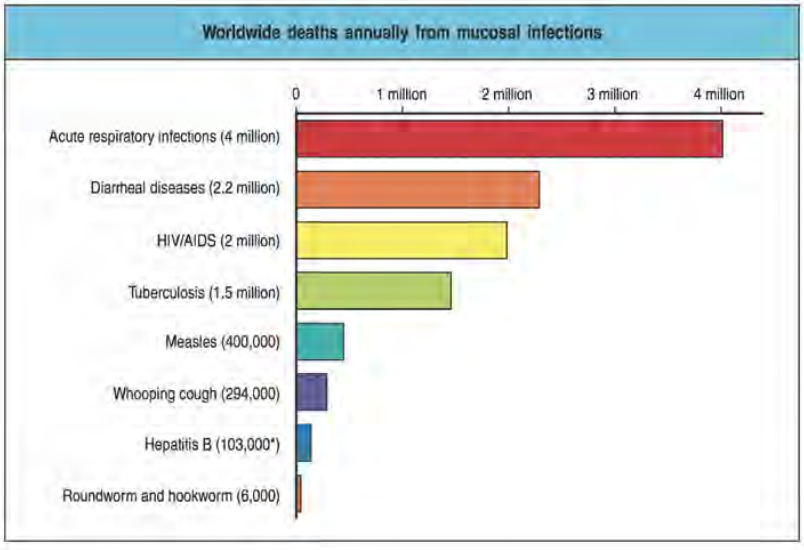


Fig. 12.2 Mucosal infections are one of the biggest health problems worldwide. Most of the pathogens that cause the deaths of large numbers of people are those of mucosal surfaces or enter the body through these routes. Respiratory infections are caused by numerous bacteria (such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, which cause pneumonia, and *Bordetella pertussis*, the cause of whooping cough) and viruses (such as influenza and respiratory syncytial virus). Diarrheal diseases are caused by both bacteria (such as the cholera bacterium *Cholera vibrio*) and viruses (such as rotaviruses). The human immunodeficiency virus (HIV) that causes AIDS enters through the mucosa of the urogenital tract or is secreted into breast milk and is passed from mother to child in this way. The bacterium *Mycobacterium tuberculosis*, which causes tuberculosis, also enters through the respiratory tract. Measles manifests itself as a systemic disease, but it originally enters via the oral/respiratory route. Hepatitis B is also a sexually transmitted virus. Finally, parasitic worms inhabiting the intestine cause chronic debilitating disease and premature death. Most of these deaths, especially those from acute respiratory and diarrheal diseases, occur in children under 5 years old in the developing world, and there are still no effective vaccines against many of these pathogens. Numbers shown are the most recent estimated figures available (*The Global Burden of Disease: 2004 Update*. World Health Organization, 2008). *Does not include deaths from liver cancer or cirrhosis resulting from chronic infection.

to commensal bacteria). As we shall see, the intestinal mucosal immune system has evolved means of distinguishing harmful pathogens from antigens in food and the natural gut microbiota. Similar issues are faced at other mucosal surfaces, such as the respiratory tract and female genital tract. Here, protective immunity against pathogens is essential but many of the antigens entering these tissues are also harmless, being derived from commensal organisms, pollen, other innocuous environmental material, and, in the lower urogenital tract, sperm.

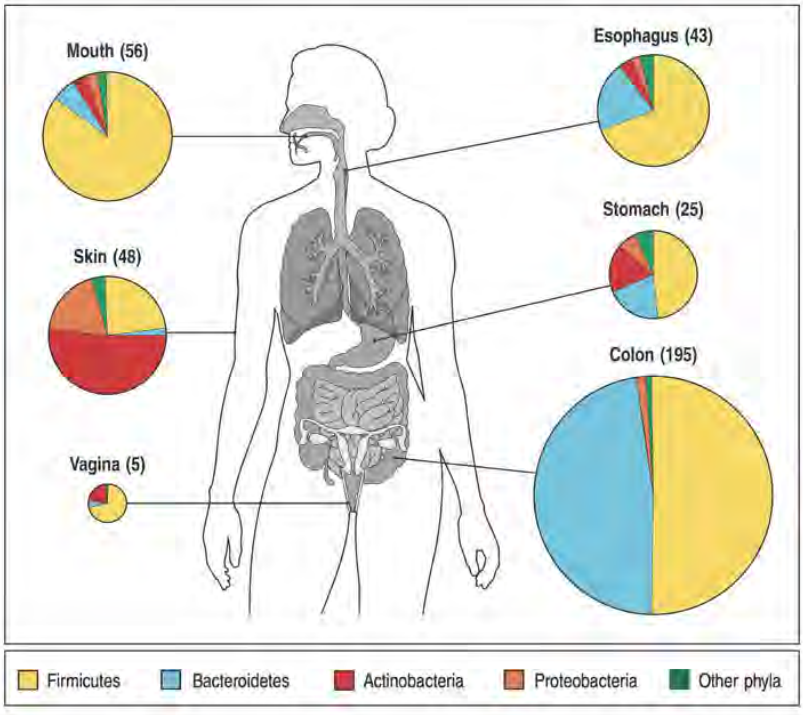


Fig. 12.3 Composition of the commensal microbiota at different mucosal surfaces in healthy humans. The different sizes of the pie-charts for different sites reflect the numbers of distinct bacterial 'species' typically present at that site. The colon contains the greatest number of different species (around 195 as estimated from individual surveys). The color key indicates the four bacterial phyla that contain the majority of commensal species. Ubiquitous commensal bacteria include *Lactobacillus* spp. (Firmicutes), *Bifidobacterium* spp. (Actinobacteria), *Bacteroides fragilis* (Bacteroidetes), and *Escherichia coli* (Proteobacteria). Adapted from Dethlefsen, L. *et al.*: *Nature* 2007, 449:811–818.

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12-2 The mucosal immune system may be the original vertebrate immune system.

From the point of view of traditional immunology, the mucosal immune system has been considered to be an unusual and relatively minor subcompartment of the immune system. In terms of size and function, this is an inaccurate description. As a result of its physiologically critical role and extent of exposure to antigens, the mucosal immune system forms the largest part of the body's immune tissues, containing approximately three-quarters of all lymphocytes and producing the majority of immunoglobulin in healthy individuals. When compared with lymph nodes and spleen (which in this chapter we will call the **systemic immune system**), the mucosal immune system has many unique and unusual features. The main distinctive features are listed in Fig. 2.4.

The mucosal immune system, in particular that of the gut, may well have been the first part of the vertebrate adaptive immune system to evolve, and it has been proposed that its evolution could be linked to the need to deal with the vast populations of commensal bacteria that coevolved with the vertebrates. Organized lymphoid tissues and immunoglobulin antibodies are first found in vertebrates in the gut of primitive cartilaginous fishes, and two important central lymphoid organs—the thymus and the avian bursa of Fabricius—derive from the embryonic intestine. It has therefore been suggested that the mucosal immune system represents the original vertebrate immune system, and that the spleen and lymph nodes are later specializations.

12-3 Cells of the mucosal immune system are located both in anatomically defined compartments and scattered throughout mucosal tissues.

Lymphocytes and other immune-system cells such as macrophages and dendritic cells are found throughout the intestinal tract, both in organized tissues and scattered throughout the surface epithelium of the mucosa and an underlying layer of connective tissue called the **lamina propria**. The

Distinctive features of the mucosal immune system	
Anatomical features	Intimate interactions between mucosal epithelia and lymphoid tissues
	Discrete compartments of diffuse lymphoid tissue and more organized structures such as Peyer's patches, isolated lymphoid follicles, and tonsils
	Specialized antigen-uptake mechanisms, e.g. M cells in Peyer's patches, adenoids, and tonsils
Effector mechanisms	Activated/memory T cells predominate even in the absence of infection
	Multiple activated 'natural' effector/regulatory T cells present
	Secretory IgA antibodies
	Presence of distinctive microbiota
Immunoregulatory environment	Active downregulation of immune responses (e.g. to food and other innocuous antigens) predominates
	Inhibitory macrophages and tolerance-inducing dendritic cells

Fig.12.4 Distinctive features of the mucosal immune system. The mucosal immune system is bigger, encounters a wider range of antigens, and encounters them much more frequently, than the rest of the immune system—what we call in this chapter the systemic immune system. This is reflected in distinctive anatomical features, specialized mechanisms for the uptake of antigen, and unusual effector and regulatory responses that are designed to prevent unwanted immune responses to food and other innocuous antigens.

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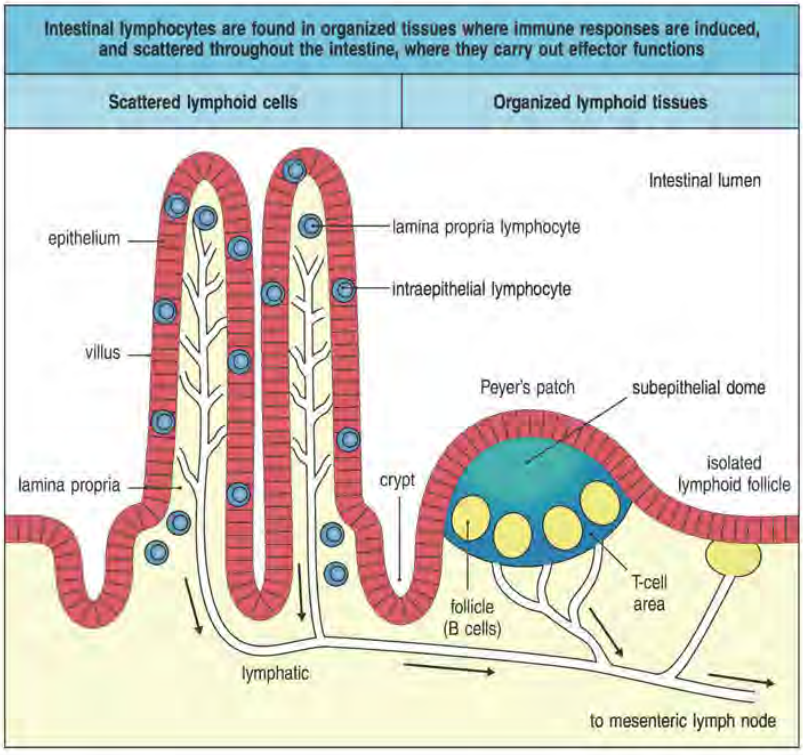


Fig. 12.5 Gut-associated lymphoid tissues and lymphocyte populations. The intestinal mucosa of the small intestine is made up of finger-like processes (villi) covered by a thin layer of epithelial cells (red) that are responsible for digestion of food and absorption of nutrients. These epithelial cells are replaced continually by new cells that derive from stem cells in the crypts. The tissue layer underlying the epithelium is called the lamina propria, and will be colored pale yellow throughout this chapter. Lymphocytes are found in several discrete compartments in the intestine, with the organized lymphoid tissues such as Peyer's patches and isolated lymphoid follicles forming what is known as the gut-associated lymphoid tissues (GALT). These tissues lie in the wall of the intestine itself, separated from the contents of the intestinal lumen by the single layer of epithelium. The draining lymph nodes for the gut are the mesenteric lymph nodes (see Fig. 12.12), which are connected to Peyer's patches and the intestinal mucosa by lymphatic vessels and are the largest lymph nodes in the body. Together, these organized tissues are the sites of antigen presentation to T cells and B cells and are responsible for the induction phase of immune responses. Peyer's patches and mesenteric lymph nodes contain discrete T-cell areas (blue) and B-cell follicles (yellow), while the isolated follicles comprise mainly B cells. Many lymphocytes are found scattered throughout the mucosa outside the organized lymphoid tissues: these are effector cells—effector T cells and antibody-secreting plasma cells. Effector lymphocytes are found both in the epithelium and in the lamina propria. Lymphatics also drain from the lamina propria to the mesenteric lymph nodes.

organized secondary lymphoid tissues in the gut comprise a group of organs known as the **gut-associated lymphoid tissues** (**GALT**), together with the draining **mesenteric lymph nodes** (Fig. 12.5). The GALT and the mesenteric lymph nodes have the anatomically compartmentalized structure typical of peripheral lymphoid organs, and are sites at which immune responses are initiated. The cells scattered throughout the epithelium and the lamina propria comprise the effector cells of the local immune response.

The GALT comprises the **Peyer's patches**, which are present in the small intestine, **isolated lymphoid follicles**, which are found throughout the intestine, and the appendix (in humans). The **palatine tonsils**, **adenoids**, and **lingual tonsils** are large aggregates of lymphoid tissue covered by a layer of squamous epithelium and form a ring, known as Waldeyer's ring, at the back of the mouth at the entrance of the gut and airways (Fig. 12.6). They often become extremely enlarged in childhood because of recurrent infections, and in the past were victims of a vogue for surgical removal. A reduced IgA response to oral polio vaccination has been seen in individuals who have had their tonsils and adenoids removed.

The Peyer's patches of the small intestine, the lymphoid tissue of the appendix (which is another frequent victim of the surgeon's knife), and the isolated lymphoid follicles are located within the intestinal wall. Peyer's patches are extremely important sites for the initiation of immune responses in the gut. Visible to the naked eye, they have a distinctive appearance, forming dome-like aggregates of lymphoid cells that project into the intestinal lumen (Fig. 12.7). There are 100–200 Peyer's patches in the human small intestine. They are much richer in B cells than the systemic peripheral lymphoid organs, each consisting of a large number of B-cell follicles with germinal centers, with small T-cell areas between and immediately below the follicles. The subepithelial dome area lies immediately beneath the epithelium and is rich in dendritic cells, T cells, and B cells. Separating the lymphoid tissues from the

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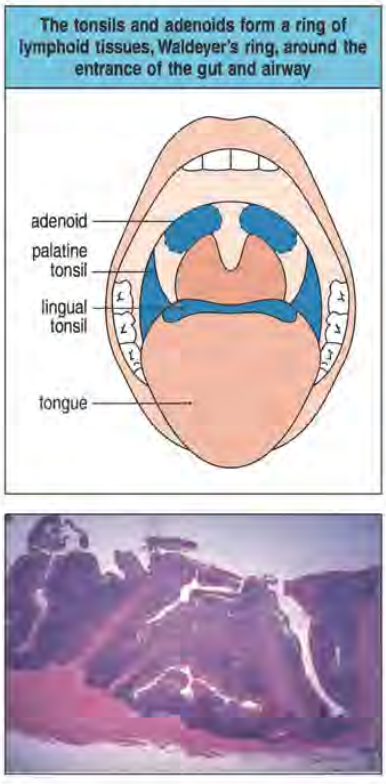


Fig. 12.6 A ring of lymphoid organs called Waldeyer's ring surrounds the entrance to the intestine and respiratory tract. The adenoids lie at either side of the base of the nose, while the palatine tonsils lie at either side of the back of the oral cavity. The lingual tonsils are discrete lymphoid organs on the base of the tongue. The micrograph shows a section through an inflamed human tonsil. In the absence of inflammation, the tonsils and adenoids normally comprise areas of organized tissue with both B-cell and T-cell areas, covered by a layer of squamous epithelium (at top of photo). The surface contains deep crevices (crypts) that increase the surface area but can easily become sites of infection. Hematoxylin and eosin staining. Magnification ×100.

gut lumen is a layer of **follicle-associated epithelium**. This contains conventional intestinal epithelial cells known as enterocytes and a smaller number of specialized epithelial cells called **microfold cells** (**M cells**), which have a folded luminal surface instead of the microvilli present on enterocytes. Unlike enterocytes, M cells do not secrete digestive enzymes or mucus and lack a thick surface glycocalyx. They are therefore directly exposed to microorganisms and particles within the gut lumen and are the route by which antigen enters the Peyer's patch from the lumen. The follicle-associated epithelium also contains lymphocytes and dendritic cells.

Several thousand isolated lymphoid follicles can be identified microscopically throughout the small and large intestines. Like Peyer's patches, these are composed of an epithelium containing M cells overlying organized lymphoid tissue, but they contain mainly B cells and develop only after birth in response to antigen stimulation due to colonization of the gut by commensal microorganisms. Peyer's patches, in contrast, are present in the fetal gut. In the gut, isolated lymphoid follicles seem to arise from small aggregates in the intestinal wall called **cryptopatches**, which contain dendritic cells and lymphoid tissue inducer cells (see Section 8-24). Peyer's patches and isolated lymphoid follicles are connected by lymphatics to the draining mesenteric lymph nodes, which are located in the connective tissue that tethers the intestine to the rear wall of the abdomen. These are the largest lymph nodes in

Fig. 12.7 A Peyer's patch and its specialized surface epithelium. Panel a: Peyer's patches are organized lymphoid tissues lying in the submucosal layer of the intestinal wall. Each comprises numerous, highly active B-cell follicles with germinal centres (GC), as well as intervening T-cell dependent areas (TDA) and a layer between the surface epithelium and the follicles known as the subepithelial dome, which is rich in dendritic cells, T cells, and B cells (see Figs 12.5 and 1.20 for schematic views of a Peyer's patch). The surface epithelium is known as the follicle-associated epithelium and is a single layer of columnar epithelial cells. Panel b: scanning electron micrograph of the follicle-associated epithelium of the mouse Peyer's patch shown boxed in (a) reveals microfold (M) cells, which lack the microvilli and the layer of mucus that is present on normal epithelial cells. Each M cell appears as a sunken area on the epithelial surface. Panel c: a higher-magnification view of the boxed area in (b) shows the characteristic ruffled surface of an M cell. M cells are the portal of entry for many pathogens and other particles. (a) Hematoxylin and eosin staining. Magnification ×100. (b) ×5000. (c) ×23,000. Source: Mowat, A., Viney, J.: *Immunol. Rev.* 1997, **156**:145–166.

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the body and play a crucial role in initiating and shaping immune responses to intestinal antigens.

The immune responses generated when antigen is recognized in one of the tissues of the GALT are quite distinct from those stimulated in lymph nodes or spleen when antigen is introduced into the skin, muscle, or bloodstream. This is because the microenvironment of the GALT has its own characteristic content of lymphoid cells, hormones, and other immunomodulatory factors. The mesenteric lymph nodes and Peyer's patches differentiate independently of the systemic immune system during fetal development, with the involvement of specific chemokines and receptors of the tumor necrosis factor (TNF) family (see Fig. 12.8; see also Section 8-24). The differences between the GALT and the systemic lymphoid organs are thus imprinted early in life and are independent of exposure to antigen.

In some species such as mice, isolated lymphoid follicles are also found in the lining of the nose, where they are called **nasal-associated lymphoid tissues** (**NALT**), and in the wall of the upper respiratory tract, when they are known as **bronchus-associated lymphoid tissues** (**BALT**). The term **mucosa-associated lymphoid tissues** (**MALT**) is sometimes used to refer collectively to all such tissues found in mucosal organs, although defined organized lymphoid tissues are not found in the nose or respiratory tract in humans unless infection is present.

Control of development of the GALT compared with systemic lymphoid tissues

	Protein required for tissue development									
Tissue	**TNFR-I**	**LT-α**	**LT-β**	**LTβR**	**TRANCE**	**IL-7R**	**α_4:β_7 integrin**	**L-selectin**	**CXCR5**	**NFκB2**
Peyer's patch	+	+	+	+	–	+	+/–	–	+/–	+
Isolated lymphoid follicle	+	+	+	+	–	+	–	–	+	+
Mesenteric lymph node	–	+	–	+	+	–	+/–	+/–	–	–
Systemic lymph node	+/–	+	+/–	+	+	–	–	+	–	+/–

Fig. 12.8 The fetal development of intestinal lymphoid tissues is controlled by a specific set of cytokines. Experiments in knockout mice show that the mesenteric lymph nodes and Peyer's patches differ from each other, and from lymph nodes in other parts of the body, in the signals that are required for their development in fetal and early neonatal life. The development of all these lymphoid tissues requires an interchange of signals between lymphoid-tissue inducer cells and local stromal cells. Signals from the stromal cells induce the lymphoid-tissue inducer cells to express lymphotoxin (LT)-α and -β subunits. These can form homotrimers (LT-α_3) or heterotrimers (LT-α_1:β_2); LT-α_1:β_2 acts on local stromal cells via the LT-β receptor, and this receptor is required for the development of all the lymphoid tissues considered here, as is the production of the LT-α subunit. Stimulation of stromal cells via the LT-β receptor leads to the expression of adhesion molecules such as VCAM-1 and the production of chemokines such as CCL19, CCL21, and CXCL13, all of which recruit lymphocytes into the developing organ, as well as more lymphoid-tissue inducer cells. Mesenteric lymph nodes are the first lymphoid tissues to develop in the fetus. Lymphoid-tissue inducer cells in these sites produce LT-α_1:β_2 in response to the TNF-family cytokine TRANCE produced by the stromal cells, but knockout experiments in mice show that the LT-β subunit is not essential for mesenteric lymph node development and that it can be replaced by another TNF-family molecule, LIGHT, which can also bind the LT-β receptor. The development of Peyer's patches is absolutely dependent on the presence of both LT-α and LT-β subunits, which are produced by lymphoid-tissue inducer cells in response to IL-7 produced by stromal cells. Lymphoid-tissue inducer cells are also uniquely recruited to Peyer's patches via their CXCR5 receptors, and the TNF receptor TNFR-I is also involved in the development of Peyer's patches but not of the other tissues shown here. In respect of LT signals, the requirements of the peripheral lymph nodes are more similar to those of the mesenteric lymph node. The differences in the requirements for LT subunits and receptors probably reflect subtle differences in the signaling pathways used in the different sites. Adhesion molecules are also involved in lymphoid tissue development. Peyer's patches develop normally in the absence of L-selectin but are partly dependent on the integrin α_4:β_7 and are entirely absent if both these proteins are lacking. Mesenteric lymph nodes also require either L-selectin or α_4:β_7 integrin, but develop normally in the absence of either. Systemic lymph nodes require only L-selectin for their development.

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12-4 The intestine has distinctive routes and mechanisms of antigen uptake.

Antigens present at mucosal surfaces must be transported across an epithelial barrier before they can stimulate the mucosal immune system. Peyer's patches and isolated lymphoid follicles are highly adapted for the uptake of antigen from the intestinal lumen, particularly those present on bacteria and viruses. The M cells in the follicle-associated epithelium are continually taking up molecules and particles from the gut lumen by endocytosis or phagocytosis (Fig. 12.9). In the case of bacteria this may involve specific recognition of the bacterial FimH protein found in type 1 pili by a glycoprotein (GP2) on the M cell. This material is transported through the interior of the cell in membrane-enclosed vesicles to the basal cell membrane, where it is released into the extracellular space—a process known as **transcytosis**. Because M cells are much more accessible than enterocytes, a number of pathogens target M cells to gain access to the subepithelial space, even though they then find themselves in the heart of the intestinal adaptive immune system (see Section 12-3).

The basal cell membrane of an M cell is extensively folded, forming a pocket that encloses lymphocytes and dendritic cells. The dendritic cells take up the transported material released from the M cells and process it for presentation to T lymphocytes. These dendritic cells are in a particularly favorable position to acquire gut antigens, and they are recruited to the follicle-associated epithelium in response to chemokines that are released constitutively by the epithelial cells. The chemokines include CCL20 (MIP-3α) and CCL9 (MIP-1γ), which bind to the receptors CCR6 and CCR1, respectively, on the dendritic cell (see Appendix IV for a listing of chemokines and their receptors). The antigen-loaded dendritic cells then migrate from the dome region to the T-cell areas of the Peyer's patch, where they meet naive, antigen-specific T cells. Together, the dendritic cells and primed T cells then activate B cells and initiate class switching to IgA. All these processes—the uptake of antigen by M cells, the migration of dendritic cells into the epithelial layer, the production of chemokines, and the subsequent migration of dendritic cells into T-cell areas—are markedly enhanced in the presence of pathogenic organisms and their products.

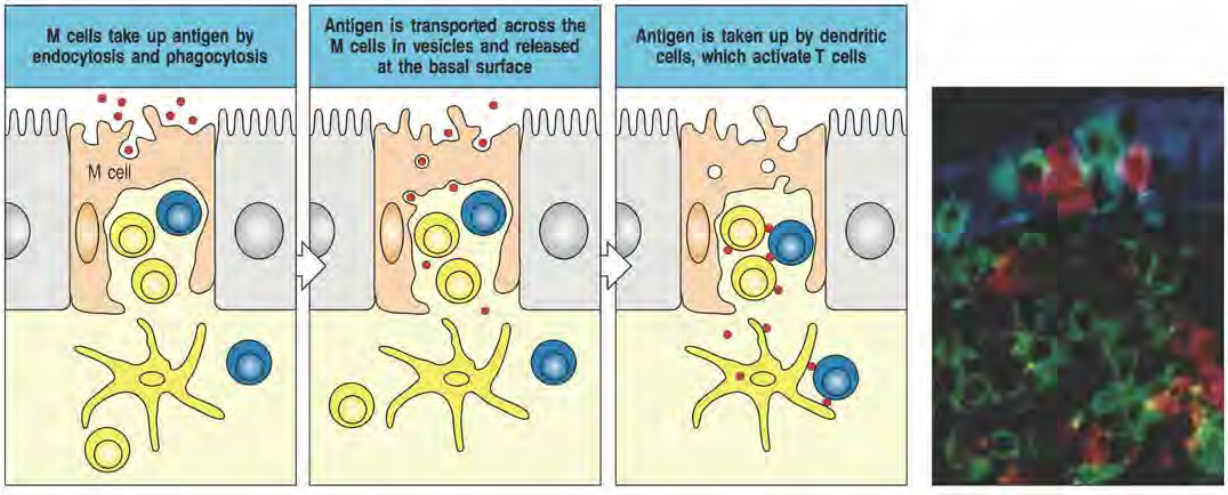


Fig. 12.9 Uptake and transport of antigens by M cells. The first three panels show uptake via M cells in the follicle-associated epithelium of Peyer's patches. These have convoluted basal membranes that form 'pockets' within the epithelial layer, allowing close contact with lymphocytes and other cells. This favors the local transport of antigens that have been taken up from the intestine by the M cells and their delivery to dendritic cells for antigen presentation. The micrograph of part of a Peyer's patch on the right shows epithelial cells (dark blue), some of which are M cells that form pockets where T cells (red) and B cells (green) accumulate. The cells have been stained with fluorescently labeled antibodies specific for individual cell types. Micrograph from Brandtzaeg, P., *et al.*: *Immunol. Today* 1999, 20:141–151.

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12-5 The mucosal immune system contains large numbers of effector lymphocytes even in the absence of disease.

In addition to the organized lymphoid organs, a mucosal surface contains enormous numbers of lymphocytes and other leukocytes scattered throughout the tissue. Most of the scattered lymphocytes have the appearance of cells that have been activated by antigen, and they comprise the effector T cells and plasma cells of the mucosal immune system. In the intestine, effector cells are found in two main compartments: the epithelium and the lamina propria (Fig. 12.10). These tissues are quite distinct in immunological terms, despite being separated by only a thin layer of basement membrane. The epithelium contains mainly lymphocytes, which in the small intestine are virtually all CD8 T cells. The lamina propria is much more heterogeneous, with large numbers of CD4 T cells and CD8 T cells, as well as plasma cells, macrophages, dendritic cells, and occasional eosinophils and mast cells. Neutrophils are rare in the healthy intestine, although their numbers increase rapidly during inflammatory disease or infection. The total number of lymphocytes in the epithelium and lamina propria probably exceeds that of most other parts of the body.

The healthy intestinal mucosa therefore displays many characteristics of a chronic inflammatory response—namely, the presence of numerous effector lymphocytes and other leukocytes in the tissues. The presence of such large numbers of effector cells is not dependent on infection by a pathogen and is unusual for a healthy, nonlymphoid tissue. It is the result of the local responses that are continually being made to the myriad of innocuous antigens that are bombarding the mucosal surfaces. As we shall see, this is a physiological process that is essential for maintaining the beneficial symbiosis between the host and its intestinal contents, and involves a balanced generation of effector and regulatory T cells. When required, however, the mucosal

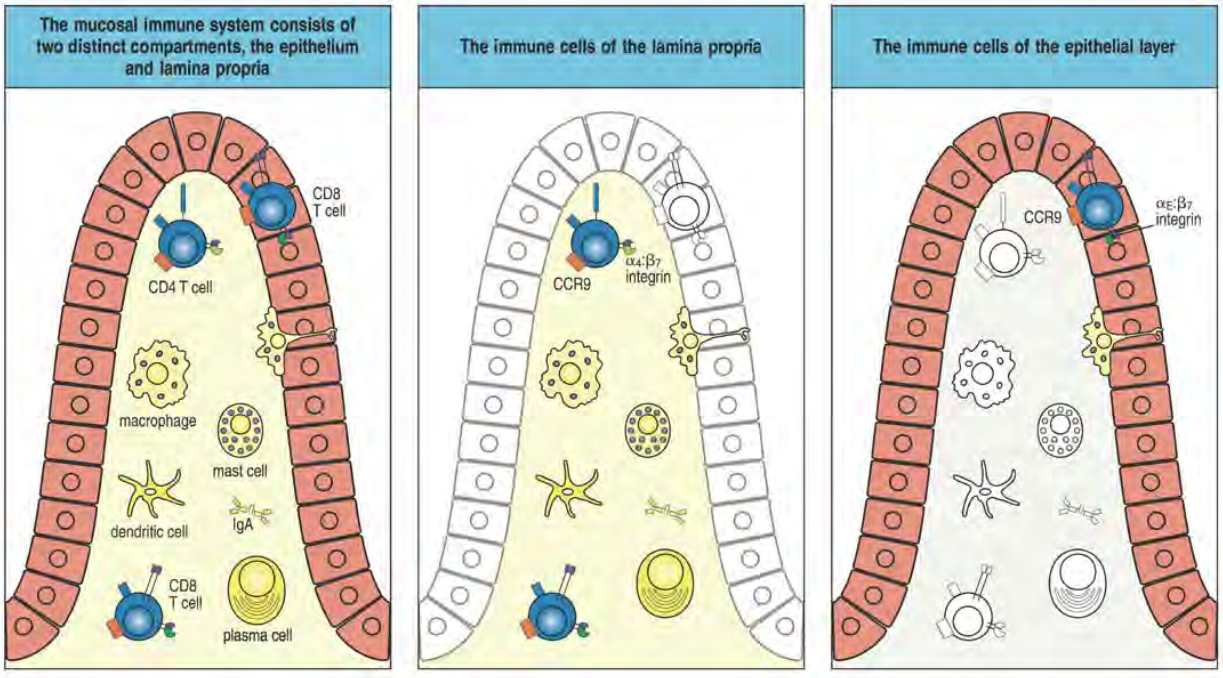


Fig. 12.10 The lamina propria and epithelium of the intestinal mucosa are discrete lymphoid compartments. The lamina propria contains a heterogeneous mixture of IgA-producing plasma cells, lymphocytes with a 'memory' phenotype (see Chapter 10), conventional CD4 and CD8 effector T cells, dendritic cells, macrophages, and mast cells. T cells in the lamina propria of the small intestine express the integrin α_4:β_7 and the chemokine receptor CCR9, which attracts them into the tissue from the bloodstream. Intraepithelial lymphocytes express CCR9 and the integrin α_E:β_7, which binds to E-cadherin on epithelial cells. They are mostly CD8 T cells, some of which express the conventional α:β form of CD8 and others the CD8α:α homodimer. CD4 T cells predominate in the lamina propria, whereas CD8 T cells predominate in the epithelium.

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immune system can be refocused on producing a full adaptive immune response to invading pathogens.

12-6 The circulation of lymphocytes within the mucosal immune system is controlled by tissue-specific adhesion molecules and chemokine receptors.

The arrival of effector lymphocytes in the mucosal surface layer is the outcome of a series of events in which the homing characteristics of lymphocytes change as they become activated. The life history of mucosal lymphocytes starts with the emergence of naive T cells and B cells from the thymus and bone marrow, respectively. At this point, the naive lymphocytes circulating in the bloodstream are not predetermined as to which compartment of the immune system they will end up in. Naive lymphocytes arriving at Peyer's patches and mesenteric lymph nodes enter them through high endothelial venules (Fig. 12.11). As in the systemic immune system, entry to peripheral lymphoid organs is controlled by the chemokines CCL21 and CCL19, which are released from the lymphoid tissues and bind the receptor CCR7 on naive lymphocytes. If the naive lymphocytes do not see their antigen, they exit from the lymphoid organ via the lymphatics and return to the bloodstream. If they encounter antigen in the GALT, the lymphocytes become activated and lose expression of CCR7 and L-selectin. This means that that they have lost their ability to home to secondary lymphoid organs, because they cannot enter them via the high endothelial venules (see Section 9-3).

Although primed lymphocytes leave the mucosal lymphoid organs in which they were activated, they travel back to the mucosa as effector cells. T and B lymphocytes initially activated in Peyer's patches, for example, leave via the lymphatics before they differentiate fully into effector cells, pass through mesenteric lymph nodes, and eventually end up in the thoracic duct. From there they circulate in the bloodstream (see Fig. 12.11) and selectively reenter the lamina propria via small blood vessels. Antigen-specific B cells are primed as IgM-producing B cells in the follicular areas of Peyer's patches and undergo switching to IgA production there, but they only differentiate fully into IgA-producing plasma cells once they have recirculated and returned to the lamina propria. Plasma cells are rarely found in Peyer's patches, and this is also true of effector T cells.

Gut-specific homing by antigen-stimulated T and B cells is determined in large part by the expression of the adhesion molecule α_4:β_7 **integrin** on the

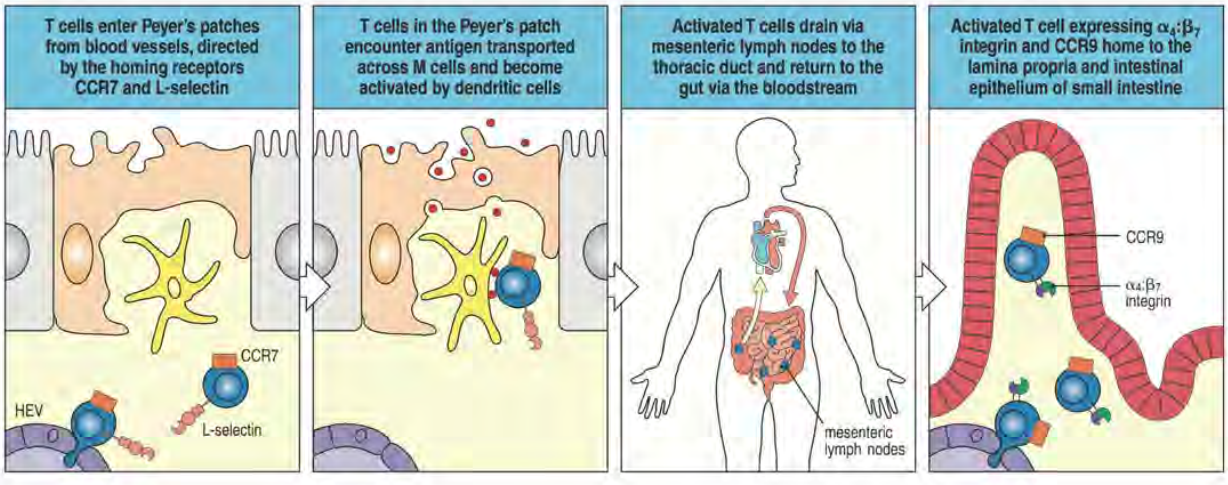


Fig. 12.11 Priming of naive T cells and the redistribution of effector T cells in the intestinal immune system. Naive T cells carry the chemokine receptor CCR7 and L-selectin, which direct their entry into Peyer's patches via high endothelial venules (HEV). In the T-cell area they encounter antigen that has been transported into the lymphoid tissue by M cells and is presented by local dendritic cells. During activation, and under the selective control of gut-derived dendritic cells, the T cells lose L-selectin and acquire the chemokine receptor CCR9 and the integrin α_4:β_7. After activation, but before full differentiation, the primed T cells exit from the Peyer's patch via the draining lymphatics, passing through the mesenteric lymph node to enter the thoracic duct. The thoracic duct empties into the bloodstream, delivering the activated T cells back to the wall of the intestine. Here T cells bearing CCR9 and α_4:β_7 are attracted specifically to leave the bloodstream and enter the lamina propria of the villus.

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lymphocytes. This binds to the mucosal vascular addressin **MAdCAM-1**, which is found mainly on the endothelial cells that line the blood vessels within the gut wall (Fig. 12.12). Lymphocytes originally primed in the gut are also lured back as a result of tissue-specific expression of chemokines by the gut epithelium. **CCL25** (TECK) is expressed by the epithelium of the small intestine and is a ligand for the receptor **CCR9** expressed on gut-homing T cells and B cells. Within the intestine there seems to be regional specialization of chemokine expression, as CCL25 is not expressed outside the small intestine and CCR9 is not required for migration of lymphocytes to the colon. However, the colon, lactating mammary gland, and salivary glands express **CCL28** (MEC, mucosal epithelial chemokine), which is a ligand for the receptor **CCR10** on gut-primed lymphocytes and attracts IgA-producing B lymphoblasts to these tissues.

Under most normal circumstances, only lymphocytes that first encounter antigen in a gut-associated secondary lymphoid organ are induced to express gut-specific homing receptors and integrins. As we shall see in the next section, these molecules are induced or 'imprinted' on T lymphocytes by intestinal dendritic cells during antigen presentation and activation. In contrast, dendritic cells from nonmucosal lymphoid tissues induce activated lymphocytes to express other adhesion molecules and chemokine receptors—for example α_4:β_1 integrin, cutaneous lymphocyte antigen (CLA), and the chemokine receptor CCR4—which direct them to tissues such as the skin (see Section 11-6). The tissue-specific consequences of lymphocyte priming in the GALT explain why vaccination against intestinal infections requires immunization by a mucosal route, because other routes, such as subcutaneous or intramuscular immunization, do not involve dendritic cells with the correct imprinting properties.

12-7 Priming of lymphocytes in one mucosal tissue can induce protective immunity at other mucosal surfaces.

Not all parts of the mucosal immune system exploit the same tissue-specific chemokines (see Section 12-6); this allows the compartmentalization of lymphocyte recirculation within the system. Thus, effector T and B cells primed in lymphoid organs draining the small intestine (mesenteric lymph nodes and Peyer's patches) are most likely to return to the small intestine; similarly, those primed in the respiratory tract migrate most efficiently back to the respiratory mucosa. This homing is obviously useful in returning antigen-specific effector

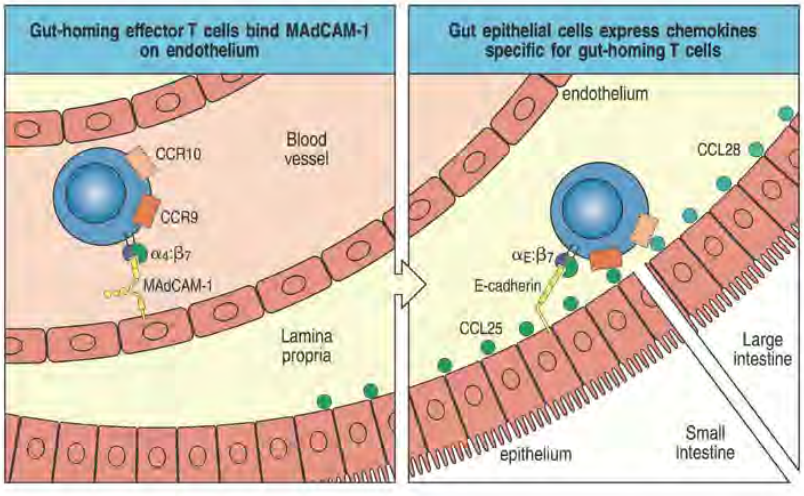


Fig. 12.12 Molecular control of intestine-specific homing of lymphocytes. Left panel: T and B lymphocytes primed by antigen in the Peyer's patches or mesenteric lymph nodes arrive as effector lymphocytes in the bloodstream supplying the intestinal wall (see Fig. 12.11). The lymphocytes express the integrin α_4:β_7, which binds specifically to MAdCAM-1 expressed selectively on the endothelium of blood vessels in mucosal tissues. This provides the adhesion signal needed for the emigration of cells into the lamina propria. Right panel: if primed in the small intestine, the effector lymphocytes also express the chemokine receptor CCR9, which allows them to respond to CCL25 (green circles) produced by epithelial cells of the small intestine; this enhances selective recruitment. Effector lymphocytes that have been primed in the large intestine do not express CCR9 but do express CCR10. This may respond to CCL28 (blue circles) produced by colon epithelial cells to fulfill a similar function. Lymphocytes that will enter the epithelial layer stop expressing the α_4:β_7 integrin and instead express the α_E:β_7 integrin. The receptor for this is E-cadherin on the epithelial cells. These interactions may help keep lymphocytes in the epithelium once they have entered it.

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cells to the mucosal organ in which they will be most effective in fighting an infection or in controlling immune responses against foreign proteins and commensals. Nevertheless, the adhesion molecule MAdCAM-1 is present on the vasculature in all mucosae. As a result, some lymphocytes that have been primed in the GALT, for example, can also recirculate as effector cells to other mucosal tissues such as the respiratory tract, urogenital tract, and lactating breast. This overlap between mucosal recirculation routes gave rise to the idea of a **common mucosal immune system**, which is distinct from other parts of the immune system. Although this is now understood to be an oversimplification, it does have important implications for vaccine development, because it enables immunization by one mucosal route to be used to protect against infection at another mucosal surface. This phenomenon has been illustrated in many experimental models, the most interesting being the ability of nasal immunization to prime immune responses in the urogenital tract against HIV. In addition, the induction of IgA antibody production in the lactating breast by natural infection or vaccination at mucosal surfaces elsewhere, such as the intestine, is an important means of generating protective immunity that is transmitted to infants by passive transfer of the antibodies in milk.

12-8 Unique populations of dendritic cells control mucosal immune responses.

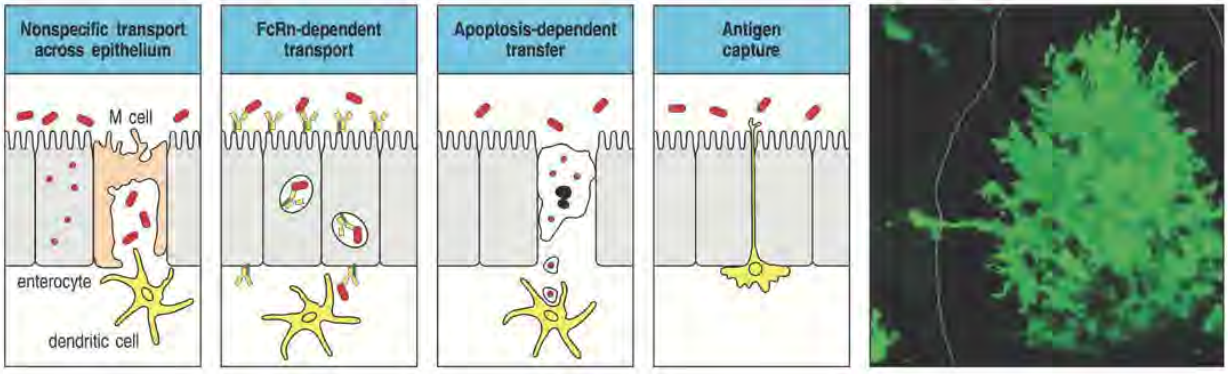
As in any compartment of the immune system, dendritic cells are the most important cell type for initiating and shaping adaptive immune responses in mucosal tissues. Dendritic cells are abundant in all mucosal tissues, being located both in the lymphoid organs and scattered throughout the mucosal surfaces. Several different subpopulations of mucosal dendritic cells have been described, many of which are distinct from their counterparts in other parts of the immune system. The nature of mucosal dendritic cells is very much determined by their local environment; in turn these cells play the major role in defining the unusual features of mucosal immune responses.

Dendritic cells are found in the Peyer's patches in two main areas. One population is found in the subepithelial dome region, and acquires antigen from M cells (see Section 12-4). Peyer's patch dendritic cells are mostly CD11b (α_M integrin)-positive and CD8α-negative, and express CCR6, the receptor for the chemokine CCL20 produced by follicle-associated epithelial cells. In resting conditions, these dendritic cells remain mostly beneath the epithelium and produce the cytokine IL-10 in response to antigen uptake, which tends to prevent the priming of T cells to become pro-inflammatory T cells.

However, during infection by a pathogen such as *Salmonella*, these dendritic cells are rapidly recruited into the epithelial layer of the Peyer's patch in response to the CCL20 that is released in increased quantities by epithelial cells in the presence of the bacteria. Bacterial products also activate the dendritic cells to express co-stimulatory molecules, allowing them to have an important role in activating pathogen-specific naive T cells to differentiate into effector cells. A distinct subset of dendritic cells that expresses CD8α but not C11b or CCR6 is also found in the T-cell area of Peyer's patches and tends to produce the pro-inflammatory cytokine IL-12.

Dendritic cells are also abundant in the wall of the intestine outside Peyer's patches, mainly in the lamina propria. How they acquire antigen across an intact epithelial barrier is not completely clear, but various ways have been proposed (Fig. 12.13). Soluble antigens such as food proteins might be transported directly across or between epithelial cells. Alternatively, there might be M cells in the surface epithelium of the mucosa outside Peyer's patches. Cells with the appearance of dendritic cells or macrophages have also been found making their way into the epithelium, or sending processes through the epithelial layer without disturbing its integrity. Such cells might acquire antigens such as bacteria from the lumen before returning with them to the lamina

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propria. Other routes by which material from the lumen can be delivered to lamina propria dendritic cells include the uptake of antibody-coated antigens by epithelial cells expressing the neonatal Fc receptor (FcRn), or the phagocytosis of antigen-containing apoptotic epithelial cells by dendritic cells (see Fig. 12.13).

The dendritic cells in the lamina propria comprise a subset with several unique properties, and they make important contributions to maintaining tolerance to harmless antigens in the intestine, especially those derived from food proteins. Most lamina propria dendritic cells express the integrin α_E:β_7 (**CD103**), and once loaded with antigen, CD103 dendritic cells leave the mucosa and migrate to the T-cell areas of mesenteric lymph nodes via the afferent lymphatics that drain the intestinal wall. In the lymph nodes they interact with naive T cells and induce the gut-homing properties that enable the T cells to efficiently return to the intestinal wall as differentiated effector T cells. The migration of CD103 dendritic cells depends on their expression of the chemokine receptor CCR7, but this expression is constitutive and does not require the presence of pathogens or other inflammatory stimuli, although it is enhanced by such agents. It is estimated that 5–10% of the mucosal dendritic cell population emigrates to the mesenteric lymph node every day in the resting intestine.

Another unusual property of CD103 dendritic cells is their production of the nonprotein signaling molecule **retinoic acid**, which is derived from the metabolism of dietary vitamin A through the action of retinal dehydrogenases. Retinoic acid production endows these dendritic cells with the unique ability to induce the expression of the gut-homing molecules CCR9 and integrin α_4:β_7 in T and B cells. Lamina propria dendritic cells respond poorly to inflammatory stimuli such as the microbial ligands for TLRs, and they produce IL-10 rather than inflammatory cytokines such as IL-12. As a result, when CD103 dendritic cells arrive in the mesenteric lymph node under resting conditions, they promote the generation of FoxP3-positive regulatory T cells (induced T_{reg} cells) from antigen-specific naive CD4 T cells (see Chapter 9 for details of the differentiation and functions of induced regulatory T cells). This process also depends on the retinoic acid produced by the dendritic cells and is assisted by transforming growth factor-β (TGF-β), a cytokine produced in abundance by intestinal cells and which promotes T_{reg} cells. Intestinal dendritic cells also produce indoleamine dioxygenase (IDO), an enzyme that catabolizes and depletes tryptophan from the environment and produces kynurenine metabolites. By some mechanism not yet understood, these actions of IDO favor the development of induced T_{reg} cells.

The anti-inflammatory behavior of mucosal CD103 dendritic cells in the healthy gut is actively promoted by factors that are constitutively produced in the mucosal environment. These include thymic stromal lymphopoietin (TSLP), retinoic acid, and TGF-β released from epithelial cells, as well as

Fig. 12.13 Capture of antigens from the intestinal lumen by mononuclear cells in the lamina propria. First panel: soluble antigens such as food proteins might be transported directly across or between enterocytes, or there might be M cells in the surface epithelium outside Peyer's patches (see Fig. 12.9 for details of transport across M cells). Second panel: enterocytes can capture and internalize antigen:antibody complexes by means of the FcRn on their surface and transport them across the epithelium by transcytosis. At the basal face of the epithelium, lamina propria dendritic cells expressing FcRn and other Fc receptors pick up and internalize the complexes. Third panel: an enterocyte infected with an intracellular pathogen undergoes apoptosis and its remains are phagocytosed by the dendritic cell. Fourth panel: mononuclear cells have been seen extending processes between the cells of the epithelium without disturbing its integrity. The cell process could pick up and internalize antigen from the gut lumen and then retract. The micrograph shows mononuclear cells, which may be dendritic cells or macrophages, (stained green with a fluorescent tag on the CD11c molecule) in the lamina propria of a villus of mouse small intestine. The epithelium is not stained and appears black, but its luminal (outer) surface is shown by the white line. A cell process has squeezed between two epithelial cells and its tip is present in the lumen of the intestine. Magnification ×200. Micrograph from Niess, J.H., *et al.*: *Science* 2005, 307:254–258.

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lipid-derived mediators such as the prostaglandin PGE_2, which is produced by stromal cells. Macrophages in the mucosa also produce IL-10 constitutively and this helps keep dendritic cells in a quiescent state, as well as maintaining the local population of regulatory T cells.

Once arrived at the mesenteric lymph node, the immunomodulatory properties of CD103 dendritic cells are further enhanced by retinoic acid produced by lymph-node stromal cells. The rapid turnover of CD103 dendritic cells allows the constant delivery of antigens from the mucosal surface to the local lymph nodes, while their immunomodulatory properties make them crucial in maintaining tolerance to harmless food proteins in the small intestine (discussed in Section 12-14). CD103 dendritic cells are also present in the large intestine and are believed to play similar roles in controlling immune responses to commensal bacteria (see Section 12-15), although this has not yet been studied in detail. Retinoic-acid-producing dendritic cells that stimulate the appearance of gut-homing molecules on T cells are also found in Peyer's patches, and may also be important for generating regulatory T cells that recognize commensal bacteria. However, CD103 dendritic cells themselves are rare in this tissue. Migratory populations of dendritic cells that continuously take up local antigens in the tissue and transport them to the draining lymph nodes are found in the lungs and at other mucosal surfaces.

Despite the overall bias toward tolerance, pro-inflammatory cells of the myeloid lineage are also present in resting lamina propria. These cells produce cytokines such as IL-6, IL-23, tumor necrosis factor-α (TNF-α), and nitric oxide, and can drive the differentiation of effector T_H17 cells and IgA switching in B cells. It has been suggested that these cells are a distinct population of dendritic cells that have been stimulated via the receptor TLR-5; they express CX3CR1, the receptor for the chemokine fractalkine (see Fig. 3.22), and do not express CD103. However, recent work indicates that these CX3CR1-positive cells in the intestine do not migrate to lymph nodes, cannot present antigen to naive T cells, and do not produce retinoic acid, and so may not be classical dendritic cells. Instead, they could be a population of macrophage-like cells whose main role is not to shape naive T-cell responses but to produce pro-inflammatory mediators during an ongoing inflammation.

12-9 The intestinal lamina propria contains antigen-experienced T cells and populations of unusual innate-type lymphocytes.

As noted above, most of the T cells in the lamina propria seem to be antigen-experienced effector cells, which have been activated by the dendritic cells discussed in the previous section. They have markers associated with effector or memory T cells, such as CD45RO in humans, and express the gut-homing markers CCR9 and α_4:β_7 integrin and receptors for pro-inflammatory chemokines such as CCL5 (RANTES). The T-cell population of the lamina propria has a ratio of CD4 to CD8 T cells of 3:1 or more, similar to that in systemic lymphoid tissues.

Lamina propria effector CD4 T cells proliferate poorly when stimulated by mitogens or antigen, but in the healthy intestine they secrete large amounts of cytokines such as interferon (IFN)-γ, IL-5, IL-17, and IL-10, even in the absence of inflammation. It is particularly noteworthy that the colon and the ileum are the only locations in the healthy body where effector T_H17 cells are found, perhaps reflecting the high bacterial load in these sites. Indeed, studies in mice show that the presence of mucosal T_H17 cells is dependent on commensal microbes, with the *Clostridium*-related segmented filamentous bacterium (SFB) playing a dominant and specific role in driving T_H17 differentiation. Cytokine-producing T_H1 and T_H2 cells are also present in the healthy lamina propria, reflecting the constant state of immune recognition of the microbiota. These lamina propria effector T cells help maintain the

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mutualistic response to commensals under resting conditions by producing cytokines such as IL-4, IL-5, IL-6, IL-21, and TGF-β, which can assist IgA production, or IL-22, which induces the secretion of antimicrobial peptides and promotes epithelial repair.

In any other situation, the presence of such large numbers of apparent effector cells would suggest the presence of a pathogen and would be likely to lead to inflammation. The fact that it does not in the healthy lamina propria is because the generation of T_H1, T_H2, and T_H17 cells is balanced by the presence of IL-10-producing, FoxP3-positive or FoxP3-negative T_{reg} cells (see Section 12-8).

During infection by a pathogen, the balance tips in favor of effector cell accumulation, either because of increased recruitment of these cells, or because the differentiation and/or functions of regulatory T cells are diminished. Now, the effector T_H1, T_H2, and T_H17 cells in the lamina propria can act without restriction to provide appropriate forms of protective immunity against the pathogen. Effector CD8 T cells are also present in the lamina propria and are capable of both cytokine production and cytotoxic activity during a protective immune response to a pathogen. Because of the pro-inflammatory cytokines they produce, T_H1 cells, T_H17 cells, and cytotoxic T cells are also the principal effectors of inflammatory disorders such as celiac disease and inflammatory bowel diseases.

The healthy lamina propria also contains unusual 'natural' effector lymphocytes. In addition to the CD1-restricted iNKT cells that are also present in the systemic immune system (see Section 6-19), there is a small subset of **mucosal invariant T cells** (**MAIT**), which express an invariant TCRα chain and respond to antigens presented by the nonclassical class I MHC molecule MR1 on B cells. MAITs are active producers of a variety of cytokines in response to unknown antigens, probably derived from commensal bacteria.

Very recently, additional and distinctive populations of non-T lymphocytes with innate effector functions have been described in human and mouse intestinal mucosa. These cells produce large amounts of IL-22, and some express the NK-cell receptors NKp44 and NKp46. They all express the transcription factor RORγT, which among other things controls the development of lymphoid tissue inducer cells. Thus they combine some of the characteristics of NK cells (see Section 3-22) and lymphoid tissue inducer cells (see Section 8-24), but seem to be distinct from both. These unusual non-T lymphocytes seem to be a major innate source of IL-22 in response to commensal microbiota. IL-22 drives the production of antimicrobial peptides in the gut and helps maintain the function of the epithelial barrier.

12-10 The intestinal epithelium is a unique compartment of the immune system.

The lymphocytes found in the intestinal epithelium—the **intraepithelial lymphocytes** (**IELs**)—are quite distinct in character from the lymphocyte population in the lamina propria or from lymphocyte populations in the systemic immune system (Fig. 12.14). There are 10–15 lymphocytes for every 100 epithelial cells in the healthy small intestine; given the enormous surface area of the mucosa, this makes the IELs one of the single largest populations of lymphocytes in the body. More than 90% of small intestinal IELs are T cells, and around 80% of these carry CD8, in complete contrast to the lymphocytes in the lamina propria. IELs are also present in the large intestine, although there are fewer relative to the number of epithelial cells and a greater proportion of CD4 T cells in comparison with the small intestine.

Like the lymphocytes in the lamina propria, most IELs have an activated appearance even in the absence of infection by a pathogen, and they contain

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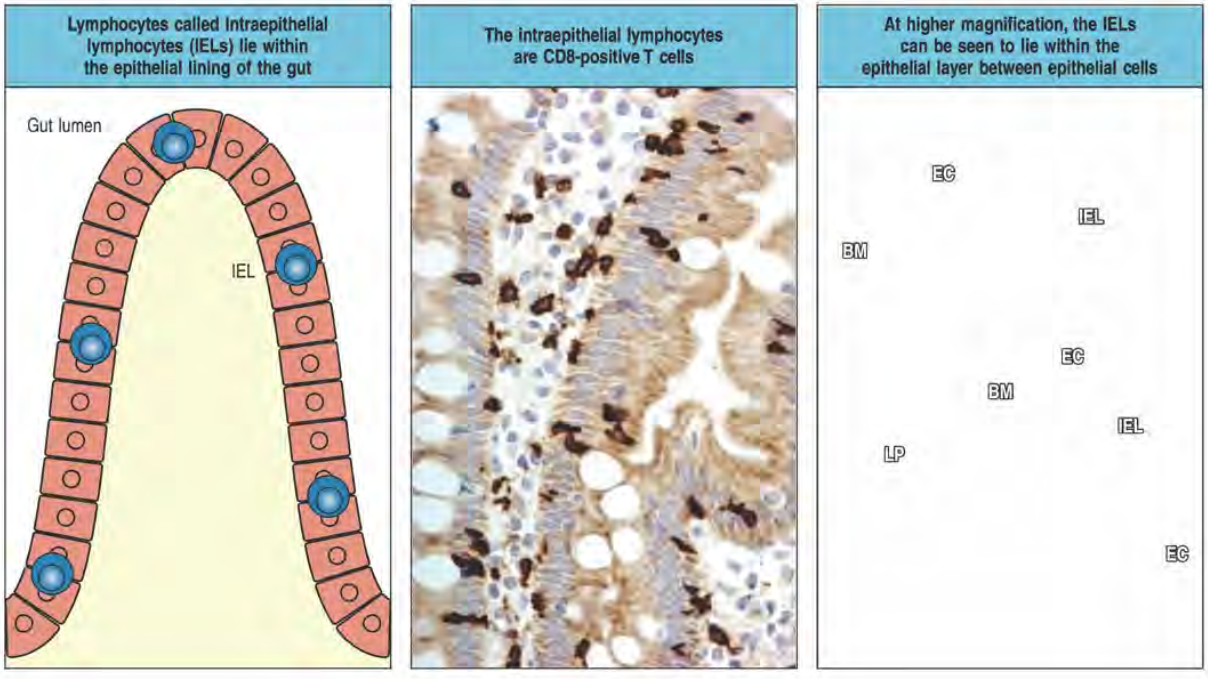


Fig. 12.14 Intraepithelial lymphocytes. The epithelium of the small intestine contains a large population of lymphocytes known as intraepithelial lymphocytes (IELs) (left panel). The micrograph in the center is of a section through human small intestine in which CD8 T cells have been stained brown with a peroxidase-labeled monoclonal antibody. Most of the lymphocytes in the epithelium are CD8 T cells. Magnification ×400. The electron micrograph on the right shows that the IELs lie between epithelial cells (EC) on the basement membrane (BM) separating the lamina propria (LP) from the epithelium. One IEL can be seen having crossed the basement membrane into the epithelium, leaving a trail of cytoplasm in its wake. Magnification ×8000.

intracellular granules containing perforin and granzymes, like those in conventional effector CD8 cytotoxic T cells. However, the T-cell receptors of most CD8 IELs show a restricted use of V(D)J gene segments, indicating that they may expand locally in response to a relatively small number of antigens. The IELs of the small intestine express the chemokine receptor CCR9, but have the α_E:β_7 integrin (CD103) on their surface instead of the α_4:β_7 integrin found on other gut-homing T cells. The receptor for α_E:β_7 integrin is E-cadherin on the surface of epithelial cells, and this interaction may assist IELs to remain in the epithelium (see Fig. 12.12).

The origin and functions of IELs are controversial. In young animals and the adults of some species, this T-cell population contains an unusually large number of γ:δ T cells. In normal adult mice and humans, however, γ:δ T cells are found in similar numbers in the gut epithelium and the bloodstream. In mice, the CD8 intraepithelial T cells can be divided into two subsets—type a and type b—depending on which form of CD8 is expressed. The relative proportions of the subsets vary with the age, mouse strain, and the number of bacteria in the intestine. Type a IELs are conventional T cells bearing α:β T-cell receptors and the CD8α:β heterodimer. They are derived from naive CD8 T cells activated by antigen in the Peyer's patches, and function as conventional class I MHC-restricted cytotoxic T cells, killing virus-infected cells, for example (Fig. 12.15, top panels). They also secrete effector cytokines such as IFN-γ and are involved in protective immunity against parasites such as *Toxoplasma gondii*.

Type b CD8 IELs comprise T cells expressing the CD8α homodimer (CD8α:α) and either an α:β or a γ:δ T-cell receptor. Some of the α:β receptors on this cell population bind nonconventional ligands, including those presented by MHC class Ib molecules (see Sections 6-17 and 6-18). All IELs express high levels of the activating C-type lectin NK receptor NKG2D (see Sections 3-21 and 3-23). This receptor can bind to two MHC-like molecules—MIC-A and MIC-B—that are expressed on intestinal epithelial cells in response to cellular injury, stress, or ligation of TLRs. The injured cells can then be recognized and killed by the IELs, a process that is enhanced by the production of IL-15

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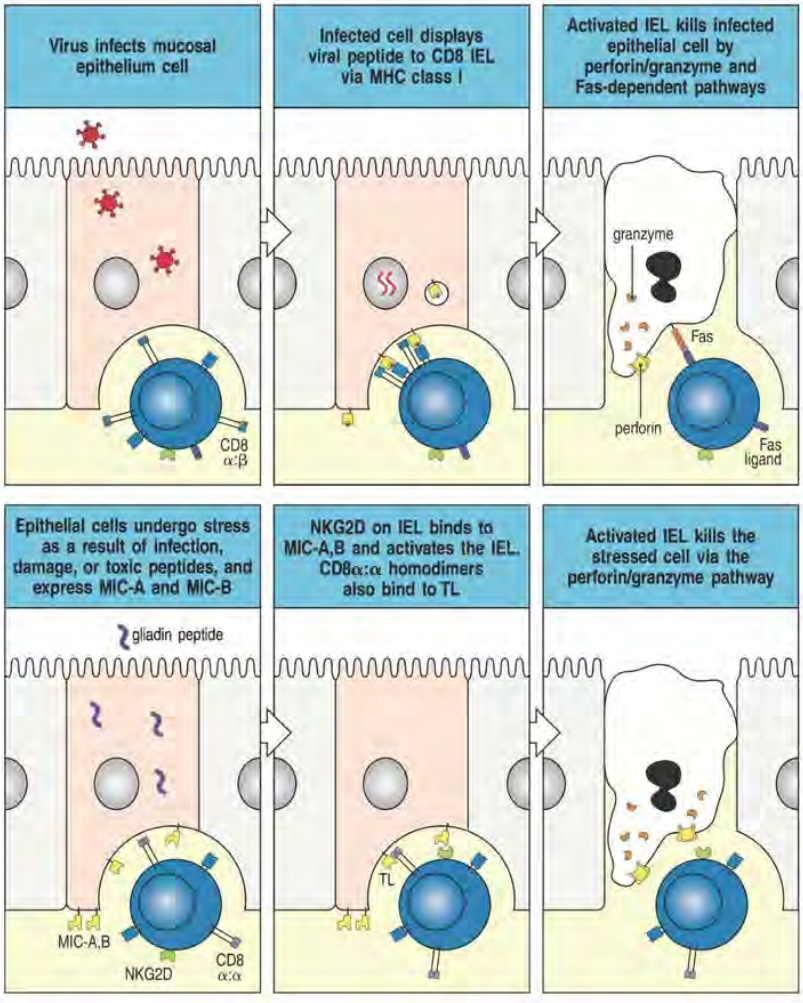


Fig. 12.15 Effector functions of intraepithelial lymphocytes. There are two main types of intraepithelial lymphocyte (IEL). As shown in the top panels, one type (type a) are conventional CD8 cytotoxic T cells that recognize peptides derived from viruses or other intracellular pathogens bound to classical MHC class I molecules on infected epithelial cells. The activated IEL recognizes specific peptide:MHC complexes by using its α:β T-cell receptor, with the CD8α:β heterodimer as co-receptor. The IEL releases perforin and granzyme, which kill the infected cell. Apoptosis of epithelial cells can also be induced by the binding of Fas ligand on the T cell to Fas on the epithelial cell. In the bottom panels, epithelial cells that have been stressed by infection or altered cell growth, or by a toxic peptide from the protein α-gliadin (a component of gluten), upregulate expression of the nonclassical MHC class I molecules MIC-A and MIC-B and produce IL-15. Neighboring type b IELs are activated by IL-15 and recognize MIC-A and MIC-B using the receptor NKG2D (see Section 3-23). They also kill the epithelial cells by releasing perforin and granzyme. These IELs carry the CD8α:α homodimer, and this protein may also contribute to their recognition of infected cells by binding directly to the nonclassical MHC class I molecule TL, encoded in the T region of the MHC, which is present on epithelial cells.

by the damaged epithelial cells. IELs can thus be considered in evolutionary terms as being at the interface between innate and adaptive immunity. Their role in the gut may be the rapid recognition and elimination of epithelial cells that express an abnormal phenotype as a result of stress or infection (Fig. 12.15, bottom panels). Gut IELs are also thought to be important in aiding the repair of the mucosa after inflammatory damage: they stimulate the release of antimicrobial peptides, thus helping to remove the source of the inflammation; they release cytokines such as keratinocyte growth factor that promote epithelial barrier function; and they suppress inflammation directly by producing cytokines such as TGF-β, which can inhibit all aspects of immune function. These repair functions have been associated particularly with the γ:δ subset of IELs, which have a similar role in skin repair.

Inappropriate or excess activation of IELs can, however, give rise to disease. MIC-A-dependent cytotoxic activity of intraepithelial T cells contributes to intestinal damage in celiac disease, which is caused by an abnormal response to the wheat protein gluten (see Section 14-18). Certain components of gluten can stimulate the production of IL-15 by epithelial cells, and celiac disease is associated with increased numbers of IELs.



Until recently, it was believed that type b IELs developed entirely within the gut. But it now seems that all IELs require the thymus for their development.

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Unlike type a IELs, however, many type b IELs seem not to have undergone conventional positive and negative selection (see Chapter 8), and express apparently autoreactive T-cell receptors. The absence of the CD8α:β heterodimer, however, means that these T cells have low affinity for conventional peptide:MHC complexes, because the CD8β chain binds more strongly than the CD8α chain to classical MHC molecules. Type b IELs therefore cannot act as self-reactive effector cells. The low affinity for self-MHC molecules is also probably the reason that these cells escape negative selection in the thymus. Instead, expression of the CD8α homodimer might enable a process of so-called **agonist selection**, in which late double-negative/early double-positive T cells are positively selected in the thymus by relatively high-affinity ligands, not unlike the selection of FoxP3-positive CD4 CD25 'natural' T_{reg} cells and iNKT cells (see Chapter 8).

The precursors of IELs exit from the thymus while they are still immature; they mature in the intestine, which may involve additional positive selection on nonclassical MHC molecules expressed on the intestinal epithelium. In some mouse strains, one of the selecting molecules in the gut is the thymus leukemia antigen (TL), which is a nonclassical MHC class I molecule that does not present peptides. TL is expressed by intestinal epithelial cells and binds the CD8α homodimer directly and with high affinity. These local differentiation events require the presence of the cytokine IL-15, which is 'trans-presented' to IELs in a complex with the IL-15 receptor present on epithelial cells.

In addition to agonist selection, type b IELs share several other properties with innate lymphocytes, including constitutive expression of genes associated with both inhibitory and activating functions. Thus the production of high levels of cytotoxic molecules, NO and pro-inflammatory cytokines and chemokines is kept in check by the constitutive expression of signaling inhibitors—the immunomodulatory cytokine TGF-β and inhibitory receptors like those found on NK cells.

12-11 Secretory IgA is the class of antibody associated with the mucosal immune system.

The dominant class of antibody in the mucosal immune system is **IgA**, which is produced locally by plasma cells present in the mucosal wall. The nature of IgA differs between the two main compartments in which it is found—the blood and mucosal secretions. IgA in the blood is mainly in the form of a monomer (mIgA) and has been produced in the bone marrow by plasma cells derived from B cells activated in lymph nodes. In mucosal tissues, IgA is produced almost exclusively as a polymer, usually as a dimer, in which the two immunoglobulin monomers are linked by a J chain (see Fig. 5.19).

The naive B-cell precursors of the IgA-secreting mucosal plasma cells are activated in Peyer's patches and mesenteric lymph nodes. Class switching of activated B cells to IgA is controlled by the cytokine TGF-β. In the human gut, class switching is T-cell dependent and occurs only in the organized lymphoid tissues, using the same molecular mechanisms as in lymph nodes and spleen (the molecular mechanisms of class switching are discussed in detail in Chapter 5, and the general consequences for immune responses in Chapter 10). The subsequent expansion and differentiation of IgA-switched B cells are driven by IL-5, IL-6, IL-10, and IL-21. Upward of 75,000 IgA-producing plasma cells are present in the normal human intestine, and 3–4 g of IgA is secreted by the mucosal tissues each day, considerably exceeding the production of all other immunoglobulin classes. This continuous production of large quantities of IgA occurs in the absence of pathogenic invasion and is driven almost entirely by recognition of the resident microbiota.

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In humans, monomeric and dimeric IgA are both found as two isotypes, IgA1 and IgA2. The ratio of IgA1 to IgA2 varies markedly depending on the tissue, being about 10:1 in blood and upper respiratory tract, about 3:2 in the small intestine and 2:3 in colon. Some common pathogens of respiratory mucosa (such as *Haemophilus influenzae*) and genital mucosa (such as *Neisseria gonorrhoeae*) produce proteolytic enzymes that can cleave IgA1, whereas IgA2 is much more resistant to cleavage. How this is related to the prevalence of IgA1 or IgA2 is uncertain. The higher proportion of plasma cells secreting IgA2 in the large intestine might be because the high density of commensal microorganisms at this site drives the production of cytokines that cause selective class switching.

After activation and differentiation, the resulting IgA-expressing B lymphoblasts express the mucosal homing integrin α_4:β_7, as well as the chemokine receptors CCR9 and CCR10, and localize to mucosal tissues by the mechanisms discussed in Section 12-6. Once in the lamina propria, the B cells undergo final differentiation into plasma cells, which synthesize IgA dimers and secrete them into the subepithelial space (Fig. 12.16). To reach its target antigen in the gut lumen, the IgA has to be transported across the epithelium. This is done via immature epithelial cells located at the base of the intestinal crypts, which constitutively express the **polymeric immunoglobulin receptor** (**pIgR**), which is located on their basolateral surfaces. This receptor has a high affinity for J-chain-linked polymeric immunoglobulins such as dimeric IgA and pentameric IgM, and transports the antibody by transcytosis to the luminal surface of the epithelium, where it is released by proteolytic cleavage of the extracellular domain of the receptor (see Fig. 12.16). Part of the cleaved pIgR remains associated with the IgA and is known as **secretory component** (frequently abbreviated to SC). The resulting antibody is referred to as **secretory IgA** (**SIgA**).

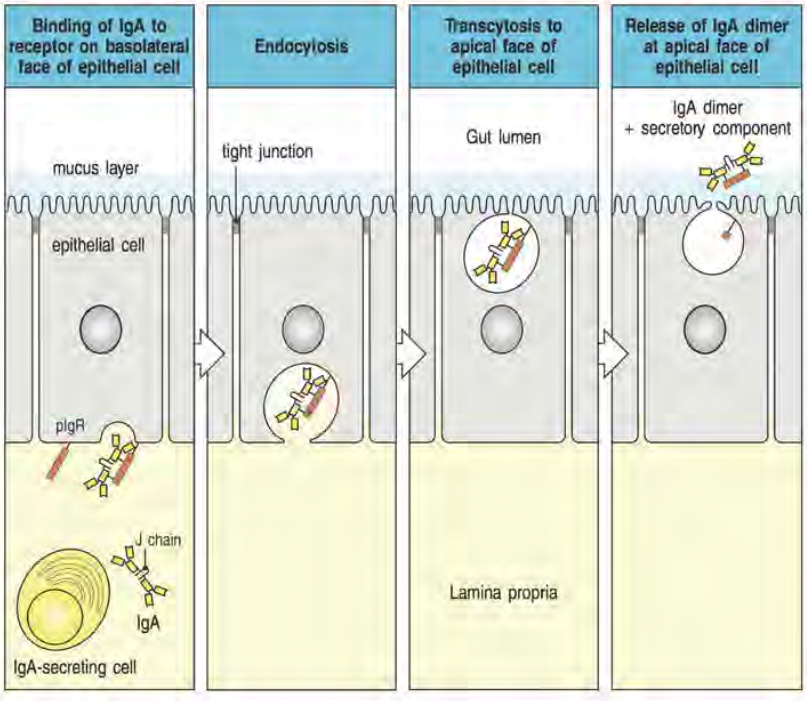


Fig. 12.16 Transcytosis of IgA antibody across epithelia is mediated by the polymeric Ig receptor (pIgR), a specialized transport protein. Most IgA antibody is synthesized in plasma cells lying just beneath epithelial basement membranes of the gut, the respiratory epithelia, the tear and salivary glands, and the lactating mammary gland. The IgA dimer linked by a J chain diffuses across the basement membrane and is bound by the pIgR on the basolateral surface of the epithelial cell. The bound complex undergoes transcytosis, by which it is transported in a vesicle across the cell to the apical surface, where the pIgR is cleaved to leave the extracellular IgA-binding component bound to the IgA molecule as the so-called secretory component. Carbohydrate on the secretory component binds to mucins in mucus and holds the IgA at the epithelial surface. The residual piece of the pIgR is nonfunctional and is degraded. IgA is transported across epithelia in this way into the lumina of several organs that are in contact with the external environment.

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In some animals there is a second route of IgA secretion into the intestine—the **hepatobiliary route**. Dimeric IgA that has not bound pIgR is taken up into the portal veins in the lamina propria, which drain intestinal blood to the liver. In the liver these small veins (sinusoids) are lined by an endothelium that allows the antibodies access to underlying hepatocytes, which have pIgR on their surface. IgA is taken up into the hepatocytes and transported by transcytosis into an adjacent bile duct. In this way, secretory IgA can be delivered directly into the upper small intestine via the common bile duct. This hepatobiliary route allows dimeric IgA to eliminate antigens that have invaded the lamina propria and have been bound there by IgA. Although highly efficient in rats, rabbits, and chickens, this route does not seem to be of great significance in humans and other primates, in whom hepatocytes do not express pIgR.

IgA secreted into the gut lumen binds to the layer of mucus coating the epithelial surface via carbohydrate determinants in secretory component. There it is involved in preventing invasion by pathogenic organisms and, just as importantly, it also has a crucial role in maintaining the homeostatic balance between the host and the commensal microbiota. IgA inhibits microbial adherence to the epithelium, an effect that is assisted by the unusually wide and flexible angle between the Fab pieces of both IgA isotypes, allowing very efficient bivalent binding to large antigens such as bacteria. Secretory IgA can also neutralize microbial toxins or enzymes.

In addition to its activities in the lumen, IgA can neutralize bacterial lipopolysaccharide and viruses it encounters inside epithelial cells, and after bacteria and viruses have penetrated across the epithelial barrier into the lamina propria. The resulting IgA:antigen complexes are then reexported into the gut lumen, from where they are excreted from the body (Fig. 12.17). Complexes containing dimeric IgA formed in the lamina propria can also be excreted via the hepatobiliary route described above. In addition to enabling the elimination of antigens, the formation of IgA:antigen complexes can enhance the uptake and transcytosis of luminal antigen by M cells and facilitate its uptake by Peyer's patch dendritic cells via their Fcα receptors (see Fig. 12.13). Secretory IgA has little capacity to activate the classical pathway of complement or to act as an opsonin, and so does not induce inflammation.

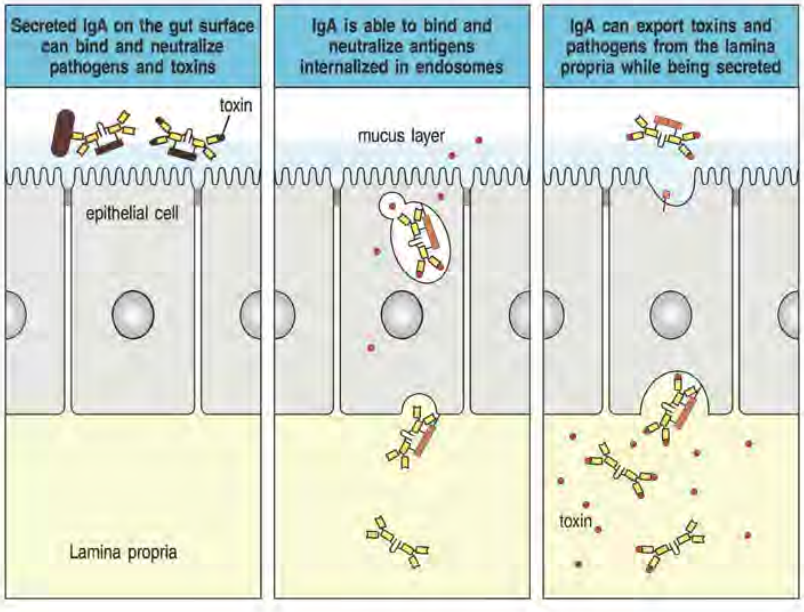


Fig. 12.17 Mucosal IgA has several functions in epithelial surfaces. First panel: IgA adsorbs on the layer of mucus covering the epithelium, where it can neutralize pathogens and their toxins, preventing their access to tissues and inhibiting their functions. Second panel: antigen internalized by the epithelial cell can meet and be neutralized by IgA in endosomes. Third panel: toxins or pathogens that have reached the lamina propria encounter pathogen-specific IgA in the lamina propria, and the resulting complexes are reexported into the lumen across the epithelial cell as the dimeric IgA is secreted.

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This means it can limit the penetration of microbes into the mucosa without risking inflammatory damage to these fragile tissues, something that is beneficial during infection by intestinal pathogens. In addition, by restricting uptake of microbes from the intestinal lumen and by facilitating antigen uptake by noninflammatory dendritic cells in the GALT, secretory IgA is crucial to the beneficial symbiosis between an individual and their gut commensal bacteria (see Section 12-15).

In mice, unlike humans, a significant proportion of intestinal IgA is derived from T-cell-independent B-cell activation and class switching (see Section 10-1), which depends on activation of the innate immune system by the products of commensal microbes and may result from the direct interaction between B cells and dendritic cells in solitary lymphoid follicles. This antibody production seems to involve lymphocytes of the B-1 subset (see Section 8-28), which arise from precursor B cells in the peritoneal cavity and migrate to the intestinal wall in response to microbial constituents such as lipopolysaccharide. Once in the mucosa, TGF-β-dependent class switching to IgA occurs under the influence of factors present in the local environment. In addition to TGF-β, these include IL-6, retinoic acid, and the TNF-family members BAFF (B-cell activating factor of the TNF family) and APRIL (a proliferation-inducing ligand), which bind to TACI (transmembrane activator and calcium-modulator and cyclophilin ligand interactor) on B cells, substituting for signals otherwise supplied by CD4 helper T cells (see Section 10-4). Intestinal epithelial cells can produce BAFF and APRIL, but the major source seems to be a specialized population of local dendritic cells that also produce nitric oxide (NO) and TNF-α, both of which assist in the processing and activation of TGF-β.

The IgA antibodies produced in these T-cell-independent responses are of limited diversity and of generally low affinity, with little evidence of somatic hypermutation. They are nevertheless an important source of 'natural' antibodies directed at commensal bacteria. As yet, there is little evidence for this source of IgA in humans, in whom all secretory IgA responses involve somatic hypermutation and seem to be T-cell dependent. The enzyme activation-induced cytidine deaminase (AID), which is essential for class switching (see Chapter 5), cannot be detected in human intestinal lamina propria, indicating that class switching is unlikely to occur there. Nevertheless, its occurrence in lamina propria B cells in mice may offer a glimpse into the evolutionary history of specific antibody responses in the mucosa, and might indicate pathways that could be activated when T-cell dependent IgA production is compromised in humans, as it is in AIDS.

12-12 IgA deficiency is common in humans but may be compensated for by secretory IgM.

Selective deficiency of IgA production is the commonest primary immune deficiency in humans, occurring in about 1 in 500 to 1 in 700 individuals in populations of Caucasian origin, although it is somewhat rarer in other ethnic groups. A slightly higher incidence of respiratory infections, atopy (a tendency to make allergic reactions to harmless environmental antigens), and autoimmune disease has been reported in older people with IgA deficiency. However, most individuals with IgA deficiency are not overly susceptible to infections unless there is also a deficiency in IgG2 production. The dispensability of IgA probably reflects the ability of IgM to replace IgA as the predominant antibody in secretions, and increased numbers of IgM-producing plasma cells are indeed found in the intestinal mucosa of IgA-deficient people. Because IgM is a J-chain-linked polymer, IgM produced in the gut mucosa is bound efficiently by the pIgR and is transported across epithelial cells into the gut lumen as secretory IgM. The importance of this back-up mechanism

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has been shown in knockout mice. Animals lacking IgA alone have a normal phenotype, but those lacking the pIgR are susceptible to mucosal infections. They also show increased penetration of commensal bacteria into tissues and a consequent systemic immune response to these bacteria. Genetic absence of the pIgR has never been reported in humans, suggesting that such a defect is lethal.

Summary.

The mucosal tissues of the body such as the intestine and respiratory tract are exposed continuously to enormous amounts of different antigens, which can be either pathogenic invaders or harmless materials such as foods and commensal organisms. Potential immune responses to this antigen load are controlled by a distinct compartment of the immune system, the mucosal immune system, which is the largest in the body and possesses many unique features. These include distinctive routes and processes for the uptake and presentation of antigens, exploiting M cells to transport antigens across the epithelium of Peyer's patches, and a unique subset of retinoic acid-producing, CD103-positive dendritic cells that imprint the T and B cells they activate with gut-homing properties. They also favor the generation of FoxP3-positive T_{reg} cells in the normal gut. Lymphocytes primed in the mucosa-associated lymphoid tissues acquire specific homing receptors, allowing them to redistribute preferentially back to mucosal surfaces as effector cells. Exposure to antigen outside the mucosal immune system cannot reproduce these effects. The mucosa-associated lymphoid tissues also generate different effector responses from those in other parts of the body, including unique forms of innate immunity. The adaptive immune response in mucosal tissues is characterized by the production of secretory dimeric IgA, and by the presence of distinctive populations of effector T cells whose functional and phenotypic properties are highly influenced by their anatomical location.

The mucosal response to infection and regulation of mucosal immune responses.

The major role of the mucosal immune response is defense against infectious agents, which include all forms of microorganisms from viruses to multicellular parasites. This means that the host must be able to generate a wide spectrum of immune responses tailored to meet the challenge of individual pathogens, and it is equally unsurprising that many microbes have evolved means of adapting to and subverting the host response. To ensure an adequate response to pathogens, the mucosal immune system needs to be able to recognize and respond to any foreign antigen, but it must not produce the same effector response to a harmless antigen (from food or commensals) as it would to a pathogen. A major role of the mucosal immune system is to balance these competing demands, and how it does this will be the focus of this part of the chapter.

12-13 Enteric pathogens cause a local inflammatory response and the development of protective immunity.

Despite the array of innate immune mechanisms in the gut, and stiff competition from the indigenous microbiota, the gut is the most frequent site of infection by pathogenic organisms. These include many viruses, enteric bacteria such as *Helicobacter pylori*, *Salmonella* and *Shigella* species, protozoans

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such as *Entamoeba histolytica*, and multicellular helminth parasites such as tapeworms and pinworms (Fig. 12.18). These pathogens cause disease in many ways, but certain common features of infection are crucial to understanding how they stimulate a productive immune response by the host. The key to this in the gut, as elsewhere in the body, is the activation of the innate immune system, one of whose functions is to stimulate the expression of co-stimulatory molecules on local dendritic cells, thus overcoming the inherent unresponsiveness that characterizes these cells in the healthy intestine and favoring the generation of an appropriate adaptive immune response.

The effector mechanisms of the innate immune system can themselves eliminate most intestinal infections rapidly and without significant spread beyond

Intestinal pathogens and human disease	
Bacteria	
Salmonella enterica Typhi	Typhoid fever
Salmonella enterica Paratyphi	Enteric fever (paratyphoid)
Salmonella enterica Enteritidis	Food poisoning
Vibrio cholera	Cholera
Shigella dysenteriae, flexneri, sonnei	Dysentery
Enteropathogenic *E. coli* (EPEC)	Gastroenteritis, systemic infection
Enterohemolytic *E. coli* (EHEC)	Gastroenteritis, systemic infection
Enterotoxigenic *E. coli* (ETEC)	Gastroenteritis, 'travelers diarrhea'
Enteroaggregative *E. coli* (EAEC)	Gastroenteritis, systemic infection
Yersinia enterocolitica	Gastroenteritis, systemic infection
Clostridium difficile	Necrotizing enterocolitis
Campylobacter jejuni	Gastroenteritis
Staphylococcus aureus	Gastroenteritis
Bacillus cereus	Gastroenteritis
Clostridium perfringens	Gastroenteritis
Helicobacter pylori	Gastritis, peptic ulcer, gastric cancer
Mycobacterium tuberculosis	Intestinal tuberculosis
Listeria monocytogenes	Foodborne infection
Viruses	
Rotaviruses	Gastroenteritis
Noroviruses	'Winter vomiting' disease, Epidemic gastroenteritis
Astroviruses	'Winter vomiting' disease
Adenoviruses	'Winter vomiting' disease
Parasites	
Protozoa	
Giardia lamblia	Gastroenteritis
Blastocystis hominis	Gastroenteritis (esp. in immunocompromised hosts)
Toxoplasma gondii	Gastroenteritis, systemic disease (esp. in immunocompromised hosts)
Cryptosporidium parvum	Gastroenteritis (esp. in immunocompromised hosts)
Entamoeba histolytica	Amebic dysentery + liver abscesses
Microsporidium species	Diarrheal disease
Helminths	
Ascaris lumbricoides	Roundworm infection of small intestine
Necator americanus	Hookworm infection of small intestine
Strongyloides species	Roundworm infection of small intestine
Enterobius species	Pinworm infection of large intestine
Trichinella spiralis	Trichinosis
Trichuris trichiura	Whipworm infection of large intestine
Taenia species	Tapeworm infections
Schistosoma species	Schistosomiasis: enteritis, mesenteric vein infection

Fig. 12.18 Intestinal pathogens and infectious disease in humans. Many species of bacteria, viruses, and parasites can cause disease in the human intestine.

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the intestine. Pattern-recognition receptors such as the TLRs are important in this process (see Chapter 3), and are expressed on both inflammatory cells and intestinal epithelial cells. Epithelial cells bear TLRs on both their apical and basal surfaces, which allows them to sense bacteria in the gut lumen and those that have penetrated across the epithelium. For example, TLR-5 on gut epithelial cells enables them to recognize flagellin (the protein of which bacterial flagella are made), and mutant mice that lack this receptor show increased susceptibility to infection by *Salmonella*. Epithelial cells also carry TLRs in intracellular vacuoles that can detect extracellular pathogens and their products that have been internalized by endocytosis (Fig. 12.19).

Epithelial cells also have intracellular sensors that can react to microorganisms or their products that enter the cytoplasm (see Fig. 12.19). These sensors include the nucleotide-binding oligomerization domain proteins NOD1 and NOD2, which are related to the TLRs and induce activation of NFκB (the details of this pathway are discussed in Section 3-8 and Figs 3.13 and 3.15). NOD1 recognizes a diaminopimelic acid-containing peptide that is found only in the cell walls of Gram-negative bacteria; NOD2 recognizes a muramyl dipeptide found in the peptidoglycans of most bacteria, and epithelial cells defective in NOD2 are less resistant to infection by intracellular bacteria. Mice lacking NOD2 also show increased translocation of bacteria across the epithelium and out of Peyer's patches. The Paneth cells of the small intestinal epithelium respond very effectively to TLR and NOD2 stimulation by producing antimicrobial peptides such as defensins (see Section 2-4), and mice lacking NOD2 or defensins are more susceptible to developing intestinal inflammation. A defect in recognition of the commensal microbiota by NOD2 also seems to be important in Crohn's disease, as up to 25% of patients carry a mutation in the *NOD2* gene that renders the NOD2 protein nonfunctional (see Section 15-23).

Ligation of TLRs or NOD proteins in epithelial cells stimulates the release of antimicrobial peptides, the production of cytokines such as IL-1 and IL-6, and the production of chemokines. The chemokines include CXCL8, which is a potent neutrophil chemoattractant (see Fig. 3.22), and CCL2, CCL3, CCL4, and CCL5, which attract monocytes, eosinophils, and T cells out of the blood. Stimulated epithelial cells also increase their production of the chemokine CCL20, which attracts immature dendritic cells resident in the tissue (see Section 12-4). In this way, the onset of infection triggers an influx of inflammatory cells and lymphocytes into the mucosa from the bloodstream, and the positioning of dendritic cells where they can capture antigen, all of which aids in the induction of a pathogen-specific adaptive immune response.

Epithelial cells also express NLRP3, a member of the NOD-like receptor family. NLRP3 is activated in response to various TLR ligands and by-products of cell injury such as ATP and reactive oxygen species, and forms part of the inflammasome, a protein complex in which caspase-1 becomes activated

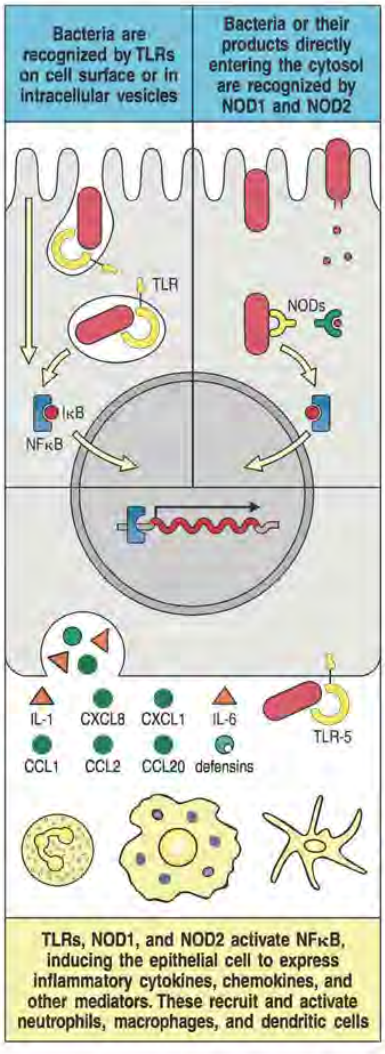


Fig. 12.19 Epithelial cells have a crucial role in innate defense against pathogens. TLRs are present in intracellular vesicles or on the basolateral or apical surfaces of epithelial cells, where they recognize different components of invading bacteria. NOD1 and NOD2 pattern-recognition receptors are found in the cytoplasm and recognize cell-wall peptides from bacteria. Both TLRs and NODs activate the NFκB pathway (see Fig. 3.13), leading to the generation of pro-inflammatory responses by epithelial cells. These include the production of chemokines such as CXCL8, CXCL1 (GROα), CCL1, and CCL2, which attract neutrophils and macrophages, and CCL20 and β-defensin, which attract immature dendritic cells in addition to possessing antimicrobial properties. The cytokines IL-1 and IL-6 are also produced and activate macrophages and other components of the acute inflammatory response. The epithelial cells also express MIC-A and MIC-B and other stress-related nonclassical MHC molecules, which can be recognized by cells of the innate immune system. IκB, inhibitor of NFκB.

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(see Section 3-8). Caspase-1 cleaves pro-IL-1 and pro-IL-18 to produce the active cytokines (see Fig. 3.16), both of which contribute to epithelial defense against bacterial invasion and can cause tissue damage if present for long periods. In acute inflammation, however, IL-18 has an important protective role, stimulating the renewal and repair of epithelial cells.

Injury and stress to the enterocytes lining the gut, and also ligation of TLR-3 by viral RNA, stimulate the expression of nonclassical MHC molecules, such as MIC-A and MIC-B (see Fig. 12.15). As discussed earlier, these proteins can be recognized by the receptor NKG2D on local cytotoxic lymphocytes, which are then activated to kill the infected epithelial cells.

12-14 The outcome of infection by intestinal pathogens is determined by a complex interplay between the microorganism and the host immune response.

Many enteric pathogens exploit host mechanisms of antigen uptake via M cells and inflammation as part of their invasive strategy. Poliovirus, reoviruses, and some retroviruses are transported through M cells by transcytosis, and initiate infection in tissues distant from the intestine after delivery into the subepithelial space. HIV may use a similar route into the lymphoid tissue of the rectal mucosa, where it first encounters and infects dendritic cells. Prions such as the causal agent of scrapie follow the same entry routes. Many of the most important enteric bacterial pathogens also gain entry through M cells. These include *Salmonella enterica* Typhi, the causative agent of typhoid fever; other *Salmonella enterica* serotypes, which are major causes of bacterial food poisoning; *Shigella* species that cause dysentery; *Yersinia pestis*, which causes plague; and *H. pylori*, which causes gastritis (inflammation of the intestine). After entry into the M cell, these bacteria produce proteins that reorganize the M-cell cytoskeleton in a manner that encourages their transcytosis. Some of the entry mechanisms used by salmonellae are shown in Fig. 12.20, and those of shigellae in Fig. 12.21.

M cells are not the only port of entry into the mucosa. Some intestinal bacteria such as *Clostridium difficile* or *Vibrio cholerae* secrete large amounts of protein toxins, enabling them to cause disease without the need to invade the epithelium. Other bacteria, such as enteropathogenic and enterohemolytic strains of *E. coli*, have specialized means of attaching to and invading epithelial cells, allowing them to cause intestinal damage and produce harmful toxins from an intracellular location. Some enteric viruses, such as rotaviruses, which cause diarrheal disease, are specialized to infect enterocytes directly.

Once delivered into the subepithelial space, pathogenic bacteria and viruses can cause a more widespread infection. Paradoxically, the host inflammatory response is an additional and often essential part of the invasive process. Many enteric bacteria stimulate the production of chemokines such as CCL20 by the epithelial cells, which recruits dendritic cells into the epithelial layer. Bacteria transcytosed through M cells are free to interact with TLRs on inflammatory cells such as macrophages and on the basal surfaces of adjacent epithelial cells. In addition, after being ingested by phagocytes, many of these microbes can be recognized by intracellular NOD1 or NLRP3 and induce NFκB and caspase-1 activation, respectively (see Section 3-8). All this stimulates the production of a cascade of inflammatory mediators, among which IL-1β and TNF-α drastically loosen the tight junctions between epithelial cells. This removes the normal barrier to bacterial invasion, allowing microorganisms to flood into the intestinal tissue from the lumen and extend the infection.

Despite their apparent benefit to the invader, it is important to remember that the principal role of the mediators and cells induced by the innate immune

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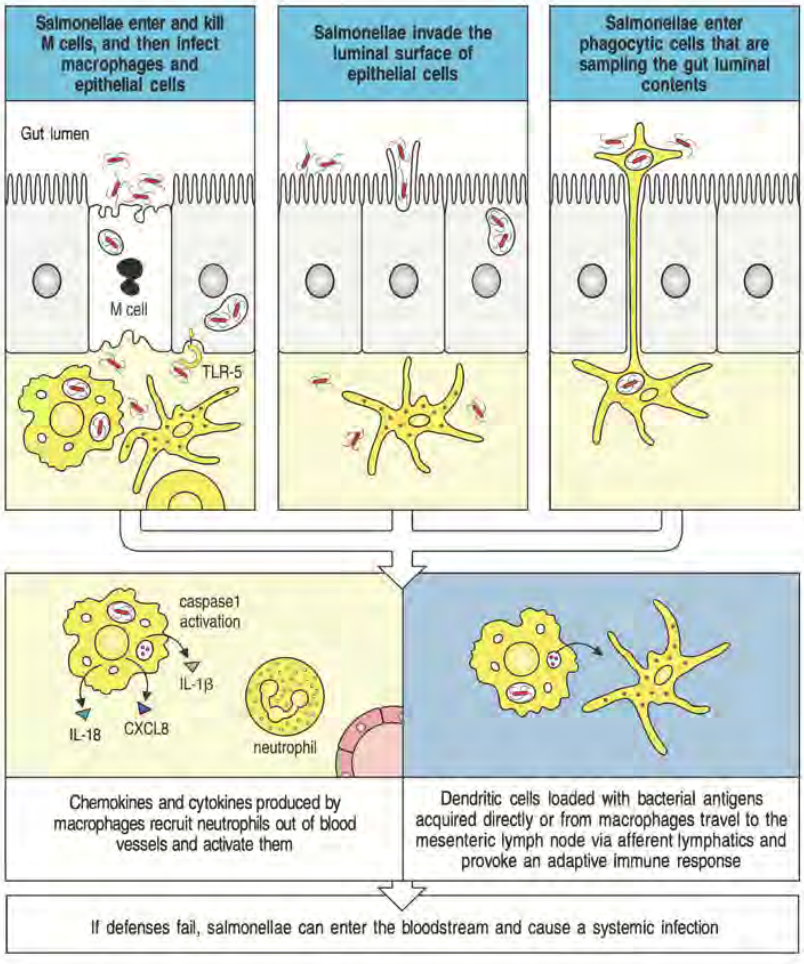


Fig. 12.20 *Salmonella enterica* serovar Typhimurium, an important cause of food poisoning, can penetrate the gut epithelial layer by three routes. In the first route (top left panel), *Salmonella* Typhimurium adheres to and enters M cells, which it then kills by causing apoptosis. Having penetrated the epithelium, it infects macrophages and gut epithelial cells. Epithelial cells express TLR-5 on their basal membrane; this binds flagellin on the salmonella flagella, activating an inflammatory response via the NFκB pathway. After uptake by macrophages in the lamina propria, invasive *Salmonella* induces caspase 1 activation, promoting production of IL-1 and IL-8. CXCL8 is also produced by the infected macrophages, and together these mediators recruit and activate neutrophils (lower left panel). Dendritic cells in Peyer's patches acquire organisms directly from M cells or from infected macrophages and present the antigens to T cells in the Peyer's patch (not shown). Salmonellae can also invade gut epithelial cells directly by adherence of their fimbriae (fine threadlike protrusions) to the luminal epithelial surface (middle panel). In the third route of entry, mononuclear phagocytes sampling the gut lumen extrude cell processes between epithelial cells. These effectively breach the epithelial layer and can be infected by salmonellae in the lumen (top right panel). Dendritic cells in villus lamina propria can acquire organisms either directly or from infected macrophages and carry them to the draining mesenteric lymph node to prime the adaptive immune response (lower right panel). If these local containment processes fail, *Salmonella* can invade beyond the intestine and its lymphoid tissues, enter the bloodstream and cause systemic infection.

response is to help initiate the adaptive immune response that will ultimately eliminate the microbe. Central to these protective effects are the cytokines IL-12, IL-18, and IL-23 produced by activated macrophages and dendritic cells, as well as IL-1, IL-6, and IL-8 produced by macrophages, dendritic cells, and epithelial cells. In infections by extracellular bacteria, IL-1, IL-6, and IL-23 drive the differentiation of T_H17 cells, which activate and recruit neutrophils; T_H17 cells also produce IL-22, which stimulates Paneth cells to produce antimicrobial peptides. In response to intracellular infections, such as those due to *Salmonella* or *Mycobacterium* species, IL-18, assisted by IL-12, drives IFN-γ production by antigen-specific T_H1 cells. In turn, this enhances the ability of the macrophage to kill bacteria it has ingested (see Section 9-29). Thus, the innate immune response to enteric bacteria has apparently opposing effects. It orchestrates a series of potent effector mechanisms aimed at eliminating infection, but these mechanisms can also be exploited by the invading organism. The fact that the protective immune response wins out in most cases is testimony to the efficiency and adaptability of the mucosal immune system.

The host–pathogen interaction is complicated further by the ability of many enteric microbes to modulate the host inflammatory response. For instance, *Yersinia* species produce Yop proteins, which can both inhibit the inflammatory response and block phagocytosis and intracellular killing of microbes by phagocytes, as well as the virulence antigen LcrV, which stimulates IL-10

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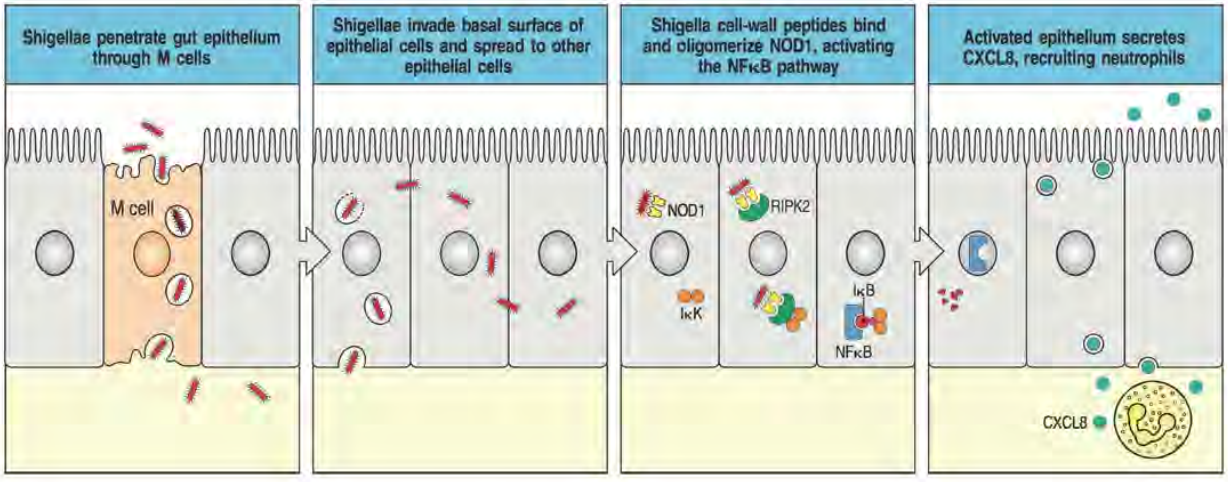


Fig. 12.21 *Shigella flexneri*, a cause of bacterial dysentery, infects intestinal epithelial cells, triggering activation of the NFκB pathway. *Shigella flexneri* binds to M cells and is translocated beneath the gut epithelium (first panel). The bacteria infect intestinal epithelial cells from their basal surface and are released into the cytoplasm (second panel). Muramyl tripeptides containing diaminopimelic acid in the cell walls of the shigellae bind to and oligomerize the protein NOD1. Oligomerized NOD1 binds the serine/threonine kinase RIPK2, which triggers activation of the NFκB pathway (see Fig. 3.15), leading to the transcription of genes for chemokines and cytokines (third panel). Activated epithelial cells release the chemokine CXCL8, which acts as a neutrophil chemoattractant (fourth panel). IκB, inhibitor of NFκB; IκK, IκB kinase.

production by binding to TLR-2. Enteropathogenic *E. coli* can inject inhibitory molecules into dendritic cells via its type III secretory system. *Salmonella enterica* Typhi creates its own safe haven within phagosomes by modifying the phagosome membrane and preventing intracellular killing (see Section 3-2). *Shigella*, in contrast, resides in the cytoplasm of epithelial cells, where it remodels the actin cytoskeleton, creating a molecular machinery that allows the bacterium to spread directly from cell to cell without exposure to the immune system. All these microorganisms also induce apoptosis of the cells they infect via the activation of caspases, and can produce factors that inhibit the activation of NFκB, thus disabling important arms of the inflammatory response as well as enhancing the spread of the pathogen (see Sections 3-7 and 3-8 for discussion of these signaling pathways). The immunomodulatory molecules produced by these bacteria are frequently essential to their ability to cause disease.

12-15 The mucosal immune system must maintain a balance between protective immunity and homeostasis to a large number of different foreign antigens.

The majority of antigens encountered by the normal intestinal immune system are not derived from pathogens, but come from food and commensal bacteria. These antigens are not only harmless but are in fact also highly beneficial to the host. They normally do not induce an inflammatory immune response, despite the fact that, like any other foreign antigens, there will be no central tolerance to them because they were not present in the thymus during lymphocyte development (see Chapter 8). The mucosal immune system has developed sophisticated means of discriminating between pathogens and innocuous antigens.

Contrary to popular belief, food proteins are not digested completely in the intestine; significant amounts are absorbed into the body in an immunologically relevant form. The default response to oral administration of a protein antigen is the development of a phenomenon known as **oral tolerance**. This is a form of **peripheral tolerance** that renders the systemic and mucosal immune systems relatively unresponsive to the same antigen. It can be demonstrated in mice by feeding them a foreign protein such as ovalbumin (Fig. 12.22). When the animals are then challenged with the antigen by a nonmucosal route, such as injection into the skin, the immune response one would expect is blunted. This suppression of systemic immune responses is long

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	Protective immunity	Mucosal tolerance	
Antigen	Invasive bacteria, viruses, toxins	Food proteins	Commensal bacteria
Primary Ig production	Intestinal IgA Specific Ab present in serum	Some local IgA Low or no Ab in serum	Local IgA No Ab in serum
Primary T-cell response	Local and systemic effector and memory T cells	No local effector T-cell response	No local effector T-cell response
Response to antigen reexposure	Enhanced (memory) response	Low or no response	Low or no mucosal or systemic response

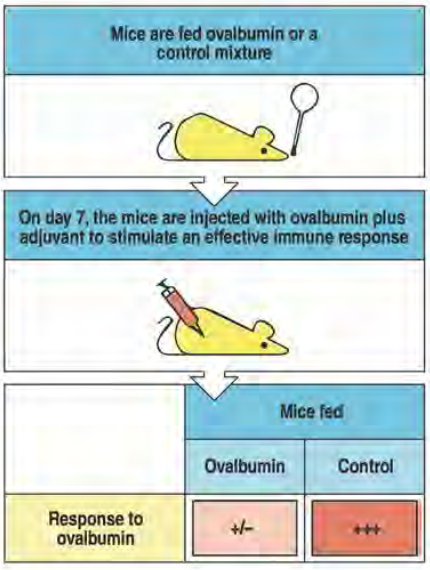


Fig. 12.22 Immune priming and tolerance are different outcomes of intestinal exposure to antigen. Left panel, the intestinal immune system generates protective immunity against antigens that are a threat to the host, such as pathogenic organisms and their products. IgA antibodies are produced locally, serum IgG and IgA are made, and the appropriate effector T cells are activated in the intestine and elsewhere. When the antigen is encountered again, there is effective memory, ensuring rapid protection. Harmless antigens such as food proteins or antigens from commensal bacteria induce tolerance, either locally, or both locally and systemically. They lack the danger signals needed to activate local antigen-presenting cells, or do not invade sufficiently to cause inflammation. In the case of food proteins, there is little or no local IgA antibody production or primary systemic antibody response, nor are effector T cells activated. Subsequent local and systemic immune responses to challenge are also specifically suppressed. This phenomenon is known as 'oral tolerance' and is shown in the right-hand panels, where it has been induced by feeding a protein such as ovalbumin to a normal mouse. First, mice are fed either ovalbumin or a different protein as a control. Seven days later, the mice are immunized subcutaneously with ovalbumin and an adjuvant; 2 weeks later, systemic immune responses such as serum antibodies and T-cell function are measured. Mice fed ovalbumin have a lower ovalbumin-specific systemic immune response than those fed the control protein. In the case of commensal bacteria, there is evidence of local IgA antibody production, but no primary systemic antibody responses, and effector T cells are not activated in either site. Profound tolerance of T-cell responses is retained thereafter, but the systemic immune system remains ignorant of commensals unless they gain access to the circulation, when a primary systemic immune response will result.

lasting and is antigen specific: responses to other antigens are not affected. A similar suppression of subsequent immune responses is observed after the administration of proteins into the respiratory tract, giving rise to the concept of **mucosal tolerance** as the usual response to such antigens delivered via a mucosal surface.

All aspects of the peripheral immune response can be affected by oral tolerance, although T-cell dependent effector responses and IgE production tend to be more inhibited than serum IgG antibody responses. Similar inhibition of systemic T-cell responses has been found in humans fed protein antigens that they have not previously encountered, although here, serum antibody responses can be completely unaffected. Thus, the systemic immune responses most susceptible to oral tolerance are those that are usually associated with tissue inflammation. Mucosal immune responses to the antigen are also downregulated. Again, this mainly affects effector T-cell responses, and low levels of secretory IgA antibodies directed at food proteins can be found in healthy humans. However, as discussed above (see Section 12-11), antibodies of this class will not lead to inflammation in the intestine. A breakdown in oral tolerance is believed to occur in celiac disease. In this condition, genetically susceptible individuals generate IFN-γ-producing, CD4 T-cell responses against the wheat protein gluten, and the resulting inflammation

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destroys the upper small intestine (see Section 14-18). Defective generation of oral tolerance has also been demonstrated in children with IgE-mediated food allergies.

Various mechanisms are likely to account for oral tolerance to protein antigens, including anergy, deletion of antigen-specific T cells, and the generation of regulatory T cells (see Chapters 8 and 9). Current evidence suggests that although antigen-specific FoxP3-positive T_{reg} cells can be generated in Peyer's patches after feeding protein antigens to experimental animals, the mesenteric lymph node plays the dominant role in their generation. This is because protein antigens seem to be mostly taken up by the retinoic-acid-producing CD103 dendritic cells of the lamina propria that migrate to a mesenteric lymph node and induce the generation of gut-homing T_{reg} cells (see Section 12-8). The events in the mesenteric lymph node are also essential for the suppression of systemic immune responses, but the mechanisms responsible for this are not yet understood.

As discussed in Chapters 9 and 11, regulatory T cells can act in various ways, but their production of TGF-β has been particularly associated with oral tolerance. TGF-β has many immunosuppressive properties and also stimulates B-cell switching to IgA. Together, these properties could help prevent inflammatory responses against food proteins by ensuring tolerance of effector T cells and by favoring the production of noninflammatory IgA antibodies. IL-10-producing T_{reg} cells may also be involved in oral tolerance; they are important in the equivalent tolerance that occurs to antigens introduced via the respiratory route. Other cytokines that can contribute to tolerance include IFN-γ, which is particularly important in preventing potentially harmful T_H2-driven allergic responses such as eosinophilia and IgE antibody production.

In addition to its physiological role in preventing inappropriate food-related immune responses, mucosal tolerance has proved effective as a way of preventing inflammatory disease in experimental animal models. Oral or intranasal administration of appropriate antigens has been found to prevent or even treat type 1 diabetes mellitus, experimental arthritis, encephalomyelitis, and other autoimmune diseases in animals. Clinical trials using mucosal tolerance to treat the equivalent diseases in humans have been less successful, possibly because it is much more difficult to inhibit inflammatory responses that are already under way; however, oral tolerance remains a potentially attractive means of inducing antigen-specific tolerance in clinical situations.

12-16 The healthy intestine contains large quantities of bacteria but does not generate potentially harmful immune responses against them.

We each harbor more than 1000 species of commensal bacteria in our intestine, and they are present in greatest numbers in the colon and lower ileum. Despite the fact that there are at least 10^{14} of these bacterial cells and they collectively weigh about 1 kg, for most of the time we cohabit with our intestinal microbiota in a happy symbiotic relationship known as **mutualism**. Nevertheless, they do represent a potential threat, as is shown when the integrity of the intestinal epithelium is damaged, allowing large numbers of commensal bacteria to enter the mucosa. This can occur when the blood flow to the gut is compromised by trauma, infection, or blood vessel disease, for example, or by endotoxic shock (see Sections 3-6 and 3-17). In these circumstances, normally innocuous gut bacteria, such as nonpathogenic *E. coli*, can cross the mucosa, invade the bloodstream, and cause fatal systemic infection.

The normal gut microbiota has coevolved with vertebrates over many millennia and has an essential role in maintaining health. Its members assist in the metabolism of dietary constituents such as cellulose, as well as degrading toxins and producing essential cofactors such as vitamin K_1 and short-chain

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fatty acids. By having direct effects on epithelial cells, the commensal bacteria are also essential for maintaining the normal barrier function of the epithelium. Another important property of commensal organisms is that they interfere with the ability of pathogenic bacteria to colonize and invade the gut. Commensals do this partly by competing for space and nutrients, but they can also directly inhibit the pro-inflammatory signaling pathways that pathogens stimulate in epithelial cells and that are needed for invasion.

The protective role of the commensal microbiota is dramatically illustrated by the adverse effects of broad-spectrum antibiotics. These antibiotics can kill large numbers of commensal gut bacteria, thereby creating an ecological niche for bacteria that would not otherwise be able to compete successfully. One example of a bacterium that grows in the antibiotic-treated gut and can cause a severe infection is *Clostridium difficile*; this produces two toxins, which can cause severe bloody diarrhea associated with mucosal injury (Fig. 12.23).

Several host factors help prevent commensal bacteria from invading beyond the intestine (Fig. 12.24). One of these is TLR signaling in epithelial cells and phagocytes, which is also important in protecting against intestinal inflammation. Mice lacking TLR-2, TLR-5, TLR-9, or the TLR adaptor protein MyD88 are much more susceptible to the induction of experimental inflammatory bowel diseases. The protective effects of TLR stimulation involve the epithelial cells being made more resistant to inflammation-induced damage, as well as tuning of the inflammatory responses themselves.

Commensal bacteria and their products are also essential for the normal development and function of the immune system. The scale of this effect is illustrated by **germ-free** (or **gnotobiotic**) animals, in which there is no colonization of the gut by microorganisms. These animals have marked reductions in the size of all lymphoid organs, low serum immunoglobulin levels, fewer mature T cells, and reduced immune responses, especially of the T_H1 and T_H17 types. In the intestine, Peyer's patches do not develop normally, isolated lymphoid follicles are absent, and there are severely reduced numbers of T lymphocytes in both the lamina propria and epithelium. There is also defective production of several of the mediators that normally regulate local immunity, such as antimicrobial peptides, IL7, IL-25, IL-33, and TSLP.

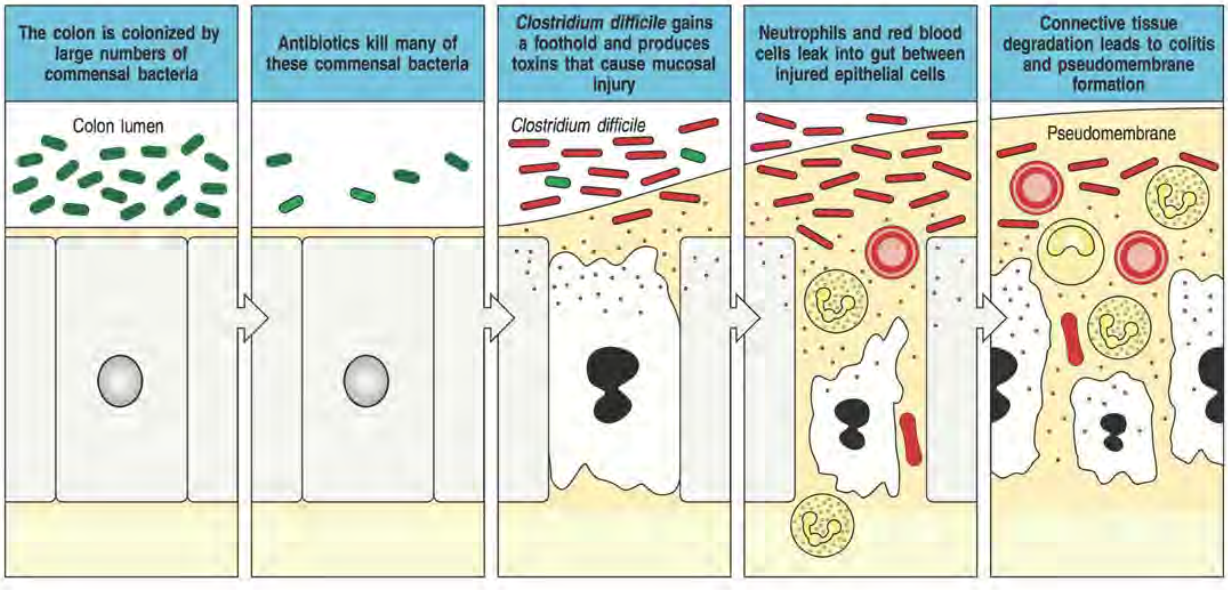


Fig. 12.23 Infection by *Clostridium difficile*. Treatment with antibiotics causes massive death of the commensal bacteria that normally colonize the colon. This allows pathogenic bacteria to proliferate and to occupy an ecological niche that is normally occupied by harmless commensal bacteria. *Clostridium difficile* is an example of a pathogen producing toxins that can cause severe bloody diarrhea in patients treated with antibiotics.

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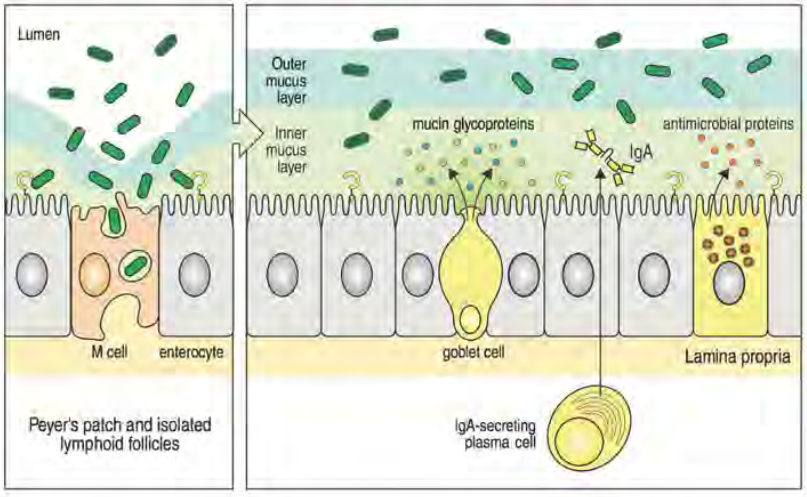


Fig. 12.24 Local responses to commensals. Several local processes ensure peaceful coexistence between the microbiota and the host, allowing the commensal organisms to be recognized by the immune system without inducing inflammation or an immune response that would eliminate them. Commensal bacteria in the lumen gain access to the immune system via M cells in Peyer's patches and isolated follicles (left panel). Uptake and presentation of these noninvasive organisms by resting dendritic cells generates IgA-switched B cells that localize in the lamina propria as IgA-producing plasma cells (right panel). The secretory IgA that is produced limits the access of commensals to the epithelium and helps prevent their penetration. This is assisted further by the presence of thick layers of mucus, which also contain mucin glycoproteins that have antibacterial properties. In addition, stimulation of pattern-recognition receptors on epithelial cells and local leukocytes induces the production of antimicrobial peptides such as defensins.

How the microbiota affects immune-system development is still being worked out; many different mechanisms are probably involved. Ligation of TLRs is likely to be important, and bacterial species have recently been identified with specific functions. Thus, polysaccharide A (PSA) from *Bacteroides fragilis* has been shown to drive the differentiation of several subsets of effector CD4 T cells, including IL-10-producing T_{reg} cells, whereas segmented filamentous bacteria (SFB) induce the appearance of T_H1,T_H17, and FoxP3-positive T cells in the mouse intestine. The effects of commensal microbes extend far beyond the intestine; for example, the incidence of several autoimmune diseases in experimental animals can be either reduced or increased in germ-free animals. In the case of type 1 diabetes, the germ-free state greatly increases the severity of the disease, perhaps because of defective development of regulatory T cells.

Commensal bacteria stimulate local IgA antibody production, and in the healthy gut there is also active suppression of local effector T-cell responses. The intestinal secretions of healthy animals contain high levels of secretory IgA directed at commensal bacteria (see Fig. 12.24). Experiments in mice that lack secretory antibodies show the importance of this response, because these animals have increased numbers of commensal bacteria penetrating the intestinal mucosa and its draining lymphoid tissues. In addition, normal individuals contain T cells that can recognize commensal bacteria, which probably accounts for the large numbers of fully differentiated T_H1 and T_H17 cells present in the resting intestine. However, as we have discussed (see Section 12-14), these effector T cells are held in check by the regulatory T cells present, producing a balanced state often called **physiological inflammation**. When strong effector T-cell responses are made against commensal bacteria, inflammatory disease such as Crohn's disease can develop (see Section 15-23).

Commensal bacteria do not induce a state of systemic immune unresponsiveness similar to the oral tolerance induced by protein antigens, and when such bacteria get into the bloodstream they stimulate a normal primary systemic immune response. They are normally confined to the gut because, unlike pathogenic bacteria, they do not possess the virulence factors necessary for penetrating the mucus layer or intact epithelium and cannot disseminate throughout the body. As a result, the only route of entry into the body for gut commensal bacteria is via M cells in Peyer's patches, with subsequent transfer to local dendritic cells. These gut-derived dendritic cells are specialized to activate naive B cells to become IgA-expressing B lymphocytes and to drive the generation of FoxP3-positive T_{reg} cells, which

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also assist the production of IgA antibodies via their secretion of TGF-β. In addition, the influence of these dendritic cells ensures that the activated T cells and B cells will migrate preferentially to the lamina propria (Fig. 12.25). This anatomically restricted response has two main outcomes. First, the locally produced IgA antibodies help limit adherence to and penetration of the epithelium by commensal bacteria. Second, T_{reg} cells accumulate in the mucosa, producing IL-10 and TGF-β, which help prevent commensal-specific T_H1 and T_H17 cells from causing overt inflammation. These effector cells do, however, retain some activity, which may also contribute to maintaining the local symbiotic relationship. For instance, T_H17-derived IL-17 stimulates the production of IL-22 and antimicrobial peptides, which helps to restrict epithelial penetration of local bacteria (see Fig. 12.24).

Several nonspecific factors assist in maintaining the host–commensal balance. Unlike pathogens, commensal bacteria do not stimulate full-blown inflammatory responses that loosen the epithelial barrier. Structural differences have been observed between the molecular patterns of commensal bacteria and of pathogens, meaning that they may induce different kinds of

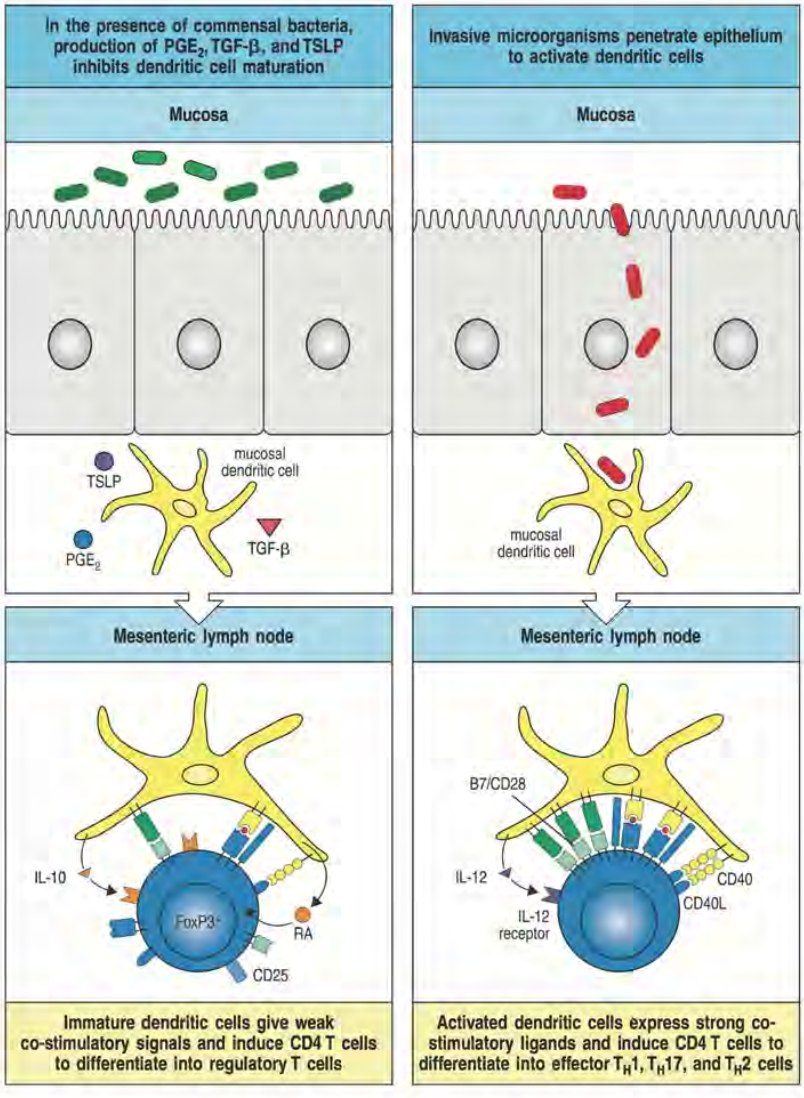


Fig. 12.25 Mucosal dendritic cells regulate the induction of tolerance and immunity in the intestine. Under normal conditions (left panels), dendritic cells are present in the mucosa underlying the epithelium and can acquire antigens from foods or commensal organisms. They take these antigens to the draining mesenteric lymph node, where they present them to naive CD4 T cells. There is, however, constitutive production by epithelial cells and mesenchymal cells of molecules such as TGF-β, thymic stromal lymphopoietin (TSLP), and prostaglandin E_2 (PGE_2), which maintain the local dendritic cells in a quiescent state with low levels of co-stimulatory molecules, so that when they present antigen to naive CD4 T cells, anti-inflammatory or regulatory T cells are generated. These recirculate back to the intestinal wall and maintain tolerance to the harmless antigens. Invasion by pathogens or a massive influx of commensal bacteria (right panels) overcomes these homeostatic mechanisms, resulting in full activation of local dendritic cells and their expression of co-stimulatory molecules and pro-inflammatory cytokines such as IL-12. Presentation of antigen to naive CD4 T cells in the mesenteric lymph node by these dendritic cells causes differentiation into effector T_H1 and T_H2 cells, leading to a full immune response.

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response through the same receptor. So although commensal bacteria are continually triggering TLRs and NODs in epithelial cells and leukocytes, they probablytriggerdifferentsignalingpathwaysfromthoseinducedbypathogens. Structural differences may also account for the fact that the endotoxin present on commensal bacteria seems unusually sensitive to neutralization by gut enzymes such as alkaline phosphatase. In addition to these inherent differences, commensal bacteria also actively inhibit the pro-inflammatory, NFκB-mediated responses induced in epithelial cells by pathogenic bacteria. This inhibition involves strategies for preventing the activation of NFκB by, for example, inhibiting the degradation of IκB (the inhibitory protein that keeps NFκB in a complex in the cytoplasm) or promoting the export of NFκB from the nucleus via the peroxisome proliferator activated receptor-γ (PPARγ) (Fig. 12.26). Finally, if commensal bacteria do cross the epithelium in small numbers, their lack of virulence factors means they cannot resist uptake and killing by phagocytic cells, and they are rapidly destroyed.

Commensal organisms can therefore remain associated with the mucosal surface without invading it or provoking inflammation and a consequent adaptive immune response. In parallel, the lack of tolerance to commensals in the systemic immune system means that the immune system will be able to generate protective immunity if they do manage to enter the body through a damaged intestine.

12-17 Full immune responses to commensal bacteria provoke intestinal disease.

It is now generally accepted that potentially aggressive T cells that can respond to commensal bacteria are present in normal animals but are usually kept in check by active regulation. If these regulatory mechanisms fail, unrestricted immune responses to commensals lead to **inflammatory bowel diseases** such as Crohn's disease (see Section 15-23). This has been demonstrated in animals with defects in immunoregulatory m echanisms involving IL-10 and TGF-β, or in which a disrupted epithelial barrier allows large numbers of commensal bacteria to penetrate. In these conditions, systemic immune responses are generated against commensal bacterial antigens such as flagellin. Strong

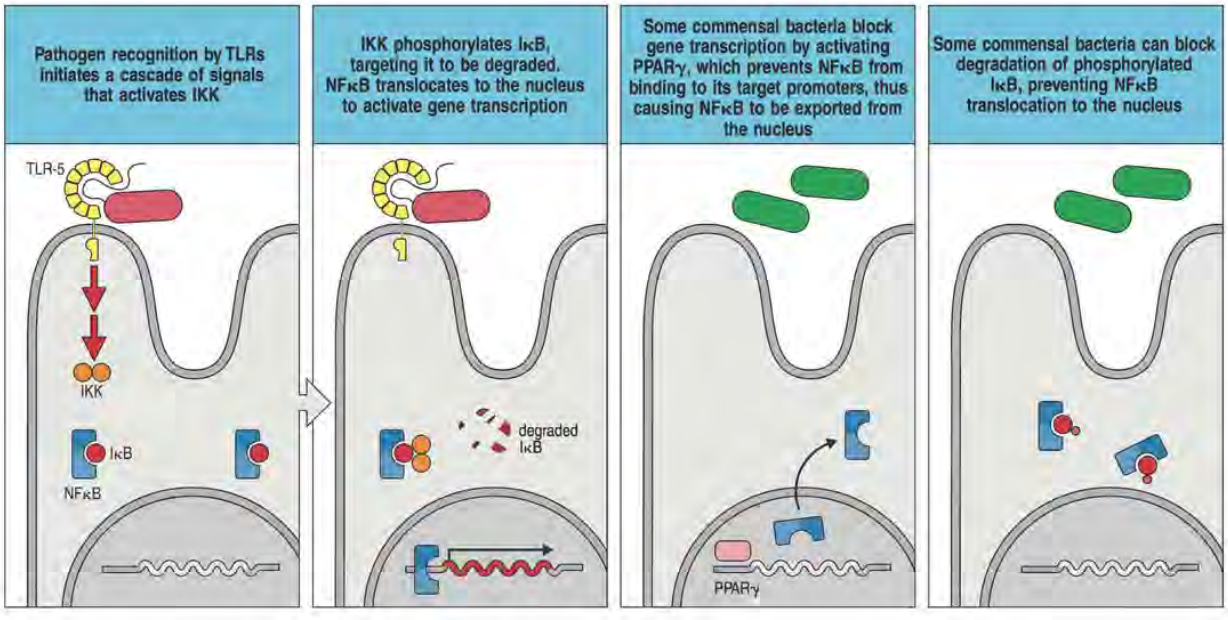


Fig. 12.26 Commensal bacteria can prevent inflammatory responses in the intestine. The pro-inflammatory transcription factor NFκB pathway is activated in epithelial cells via the ligation of TLRs by pathogens (first two panels). Commensal bacteria have been found to inhibit this pathway and thus prevent inflammation. One way is by activation of the nuclear receptor PPARγ, leading to the export of NFκB from the nucleus (third panel). Another is by blocking the degradation of the inhibitor IκB and thus retaining NFκB in the cytoplasm (fourth panel).

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inflammatory T-cell responses are also generated in the mucosa, leading to severe intestinal damage. IL-23 has a particularly important role in this process, probably mostly via its ability to drive the differentiation of T_H17 effector cells, which produce IL-17, IL-21, and IL-22. IL-23 can also assist inflammatory T_H1 responses in the intestine. These experimental results are consistent with clinical evidence for a linkage between polymorphisms in the IL-23 receptor and Crohn's disease in humans. TNF-α is also heavily involved as a final common mediator, and antibody-mediated neutralization of this cytokine is effective in treating Crohn's disease (see Fig. 12.24, right panels). In all cases, these disorders are entirely dependent on the presence of commensal bacteria, because they can be prevented by treatment with antibiotics and do not occur in germ-free animals. It is not known whether all, or only some, commensal species can provoke inflammation.

An excellent example of an inappropriate immune response against a bacterium is the gastritis associated with chronic infection by *Helicobacter pylori*. Although, strictly speaking, this is a pathogenic bacterium, it has coevolved with humans over millennia and is present in the stomachs of a large proportion of the world's population. In developing countries, more than 90% of adults may be infected for most of their lives, but even in North America and Europe up to 50% of adults are infected. *H. pylori* achieves this in the face of an ongoing host immune response, making it one of the most successful persistent infections of humans. Curiously for such a successful inhabitant of humans, *H. pylori* induces a chronic inflammatory reaction characterized by high levels of cytokines and chemokines such as IL-1, IL-6, IL-8, IL-12, IL-23, and TNF-α. Production of these cytokines is stimulated by the bacterium's virulence factors, including cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA). It is unclear whether the host obtains any benefit from the persistent colonization with *H. pylori*; indeed, the chronic infection causes gastritis, and also duodenal and gastric ulcers, and is one of the leading causes of stomach cancer in the world. It also predisposes to the development of lymphoma in mucosa-associated lymphoid tissues. Although studies in animals indicate that it is possible to induce sterilizing immunity against *H. pylori*, persistently infected humans clearly fail to induce the appropriate protective response, allowing chronic stimulation of the immune system.

The exact nature of the protective and inflammatory responses against *H. pylori* is not yet clear. Innate responses such as mucus production are key. Current evidence suggests that T_H17 cells may also be associated with clearance of *H. pylori* via the recruitment of neutrophils, whereas T_H1 cells producing IFN-γ may be responsible for the chronic gastritis; in turn, the gastritis favors bacterial survival by reducing levels of gastric acid. Although present, local and systemic antibody responses seem not to contribute to either form of immunity. *H. pylori* also actively manipulates the host immune response to favor its own survival. Not only does it employ several immune evasion strategies, but it also drives the development of induced T_{reg} cells *in vivo* by mechanisms that are as yet unknown. The T_{reg} cells selectively inhibit protective T_H17 responses and allow chronic inflammation to persist, preserving the organism's niche in the infected host.

12-18 Intestinal helminths provoke strong T_H2-mediated immune responses.

The intestines of virtually all animals and humans, except the human population of the developed world, are colonized by large numbers of helminth parasites (Fig. 12.27). Although many of these infections may be cleared rapidly by the generation of an effective response, they are also important causes of debilitating and chronic disease in humans and animals. In these circumstances, the parasite persists for long periods apparently undisturbed by the host's attempts to expel it, and causes disease by competing with the host

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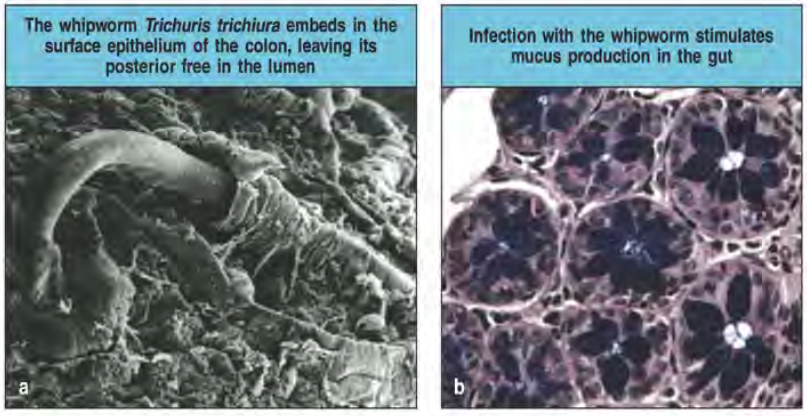


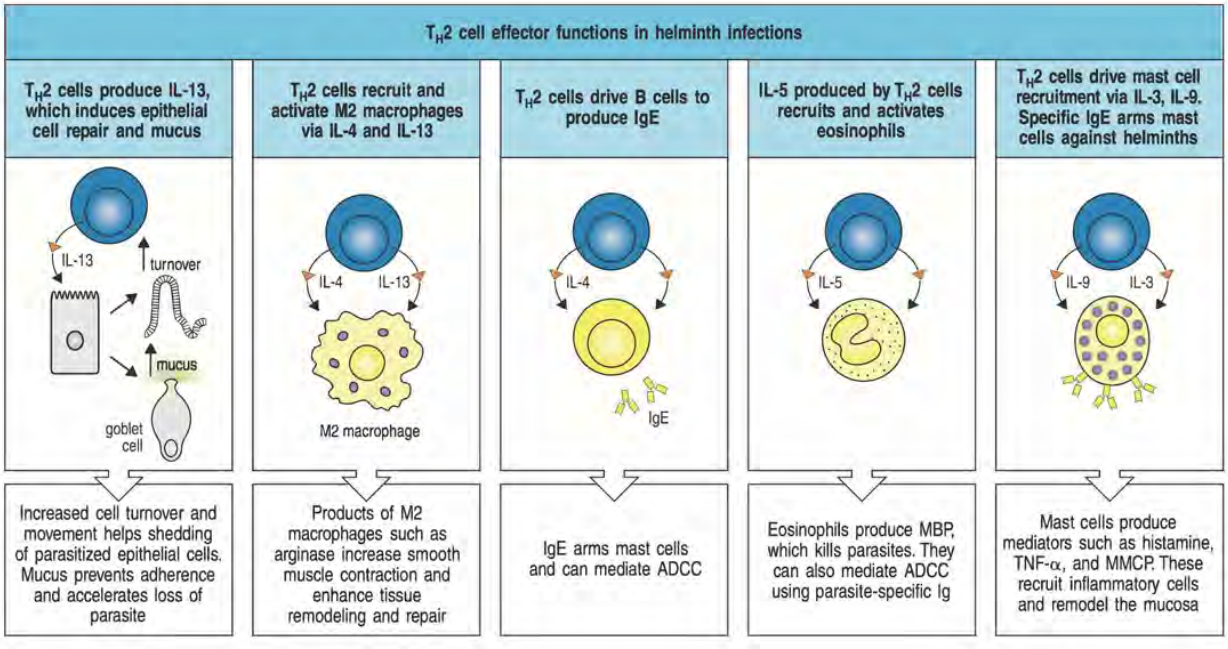
Fig. 12.27 Intestinal helminth infection. Panel a: the whipworm *Trichuris trichiura* is a helminth parasite that lives partly embedded in intestinal epithelial cells. This scanning electron micrograph of mouse colon shows the head of the parasite buried in an epithelial cell and its posterior lying free in the lumen. Panel b: a cross-section of crypts from the colon of a mouse infected with *T. trichiura* shows the markedly increased production of mucus by goblet cells in the intestinal epithelium. The mucus is seen as large droplets in vesicles inside the goblet cells and stains dark blue with periodic acid–Schiff reagent. Magnification ×400.

for nutrients, or by causing local damage to epithelial cells or blood vessels. In addition, the host immune response against these parasites can produce many harmful effects.

The exact nature of the host–pathogen interaction in helminth infections depends very much on the type of parasite involved. Some remain within the gut lumen, whereas others invade and colonize epithelial cells; others invade beyond the intestine and spend part of their life cycle in other tissues, such as liver, lung, or muscle; some are found only the small intestine, whereas others inhabit the large intestine. In virtually all cases, the protective immune response is generated by CD4 T_H2 cells, which, among other things, induce B cells to switch to the IgE isotype (Fig. 12.28), whereas a T_H1 response does not clear the pathogen and tends to produce an inflammatory reaction that damages the mucosa (Fig. 12.29, last two panels). A T_H1 response is generated when the activating dendritic cells express IL-12.

The T_H2 response is polarized by worm products acting on a variety of different cells such as basophils, dendritic cells, and epithelial cells. Dendritic

Fig. 12.28 Protective responses to intestinal helminths. Most intestinal helminths induce both protective and pathological immune responses by CD4 T cells. T_H2 responses tend to be protective, creating an unfriendly environment for the parasite, and leading to its expulsion and the generation of protective immunity (see the text for details). M2 macrophage, alternatively activated macrophage.



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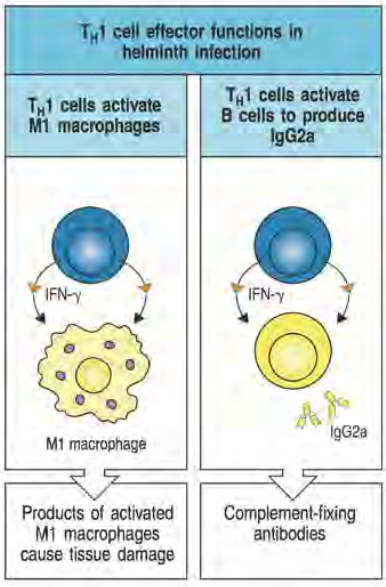


Fig. 12.29 Pathological responses to intestinal helminths. If the CD4 T-cell response to a helminth parasite is polarized to produce predominantly T_H1 effector T cells (for example, by the production of IL-12 by dendritic cells), it does not clear the pathogen (see the text for details). If not balanced by a protective T_H2 response (see Fig. 12.28), the T_H1 response leads to persistent infection and chronic intestinal pathology. M1 macrophage, classically activated macrophage.

cells are required for the presentation of helminth antigens to naive CD4 T cells, but the initial source of the IL-4 needed to drive differentiation into T_H2 effector cells is unknown. Although several cell types have been proposed as the source, including iNKT cells, mast cells, and basophils, none of these has been proved to be essential. The helminth-induced factors that drive the differentiation of naive CD4 T cells into T_H2 cells are also still a matter of debate, but seem to include the cytokines IL-25, perhaps produced by basophils, and IL-33 and TSLP produced by epithelial cells and other nonhematopoietic cells. These may act directly on the differentiating T cells, or indirectly via cells such as mast cells. Worm products and IL-25 further favor T_H2 responses by preventing IL-12 production and the generation of T_H1 cells.

The typical mucosal response to helminths shows high levels of IgE antibody, the activation of mast cells and eosinophils (see Section 10-24), activated macrophages, and tissue remodeling (see Fig. 12.28), although the precise involvement of each response depends on the type of parasite. The antihelminth response is mediated by cytokines produced by T_H2 cells. IL-13 directly enhances the production of mucus by goblet cells and increases the migration and turnover of epithelial cells (see Fig. 12.28, first panel). This is a critical component of the host response to parasitic worms. It helps to eliminate parasites that have attached to the epithelium and decreases the surface area available for colonization. Turnover increases partly because epithelial cells in the crypt respond to the loss of damaged cells from the surface epithelium by dividing more rapidly in an attempt to repair the damage. Increased epithelial cell turnover is also a direct and specific effect of the IL-13 produced by T cells, NK cells, and iNKT cells in the presence of infection. Although it makes life difficult for the parasite, increased epithelial turnover also compromises intestinal function, because the newly produced epithelial cells are immature and so are deficient in absorptive and digestive activity. Thus, the host immune response in intestinal helminths has to tread an extremely fine line, because the most efficient aspects of the protective response are also likely to be deleterious in the local environment.

IL-4 and IL-13 produced by T_H2 cells result in the differentiation of **alternatively activated macrophages** (also called **M2-type macrophages**). Unlike conventional pro-inflammatory macrophages (**M1-type macrophages**), which tend to differentiate after interaction with T_H1 cells (see Fig. 12.29), M2-type macrophages produce the enzyme arginase, which, along with other factors, increases the contractility of intestinal smooth muscle and promotes tissue remodeling and repair (see Fig. 12.28, second panel). These effects make it difficult for the parasite to attach to the epithelial surface for any length of time. IL-4 and IL-13 also stimulate the switching of B cells to IgE production (see Fig. 12.28, third panel). The IL-5 produced by T_H2 cells recruits and activates eosinophils (see Fig. 12.28, fourth panel), which can have direct toxic effects on pathogens by releasing cytotoxic molecules such as major basic protein (MBP). Eosinophils bear Fc receptors for IgG and can show antibody-dependent cell-mediated cytotoxicity (ADCC) against IgG-coated parasites (see Fig. 10.23). They also express the Fcα receptor (CD89) and degranulate in response to stimulation by secretory IgA.

The IL-3 and IL-9 produced by T_H2 cells in the mucosa recruit and activate a specialized population of mast cells known as **mucosal mast cells** (see Fig. 12.28, fifth panel). The innate cytokines IL-25 and IL-33 also activate mucosal mast cells early in a response to helminths. Mucosal mast cells differ from their counterparts in other tissues by having only small numbers of IgE receptors and producing very little histamine. When activated by cytokines, or by the binding of antigen to receptor-bound IgE, mucosal mast cells produce large amounts of preformed inflammatory mediators such as prostaglandins, leukotrienes, and several proteases, including the mucosal mast cell protease (MMCP-1) (see Chapter 10). This enzyme can remodel intestinal mucosal

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tissues by digesting the basement membrane between the epithelium and the lamina propria, and may also have direct effects on parasites. Together, the mast-cell-derived mediators increase vascular permeability, induce leukocyte recruitment, increase intestinal motility, and stimulate the production of mucus by goblet cells, all of which help to create a hostile microenvironment for the parasite. Mast cells also produce large quantities of TNF-α, which may assist in killing parasites and infected epithelial cells. TNF-α is, however, also an important cause of the inflammation and intestinal damage that occurs in such infections.

Many intestinal helminths are the ultimately adapted chronic infectious agents, having evolved sophisticated methods of persisting for long periods in the face of an ongoing immune response. They modulate the host immune response in several ways, including the production of mediators that dampen the innate inflammatory response, and the expression of decoy receptors for inflammatory cytokines and chemokines. In addition, several molecules secreted by helminths modify the differentiation of T cells, selectively encouraging the generation of T_{reg} cells at the expense of effector cells. TGF-β induced by IL-13 is crucial to this, suppressing many inflammatory responses and favoring the development of IL-10-producing T_{reg} cells. The overall effect is to limit the production and inflammatory potential of cytokines such as IFN-γ and TNF-α and to modulate effector T_H2 responses, which produces a state of persistent infection in which damage to the host is limited. These opposing immunological processes operate simultaneously in many parasitic infections, rather as we saw in the response to commensal bacteria but to a more exaggerated extent. This can result in an intestine that seems badly inflamed but can retain some physiological function, despite harboring large numbers of live, multicellular parasites.

12-19 Other eukaryotic parasites provoke protective immunity and pathology in the gut.

In addition to multicellular worms, the intestinal immune system has to contend with a variety of unicellular eukaryotic parasites, including protozoans such as *Giardia lamblia*, *Cryptosporidium parvum*, *Entamoeba histolytica*, and *Toxoplasma gondii*. *Giardia lamblia* is a widespread noninvasive waterborne microorganism that is an important cause of intestinal inflammation. Protective immunity to *G. lamblia* is associated with the production of local antibodies and infiltration of the mucosa by effector T cells, including IELs, but immunity can be inefficient, leading to chronic disease. *Cryptosporidium parvum* and *T. gondii* are usually opportunistic infections, being most commonly found in people with immune deficiencies such as AIDS. They are intracellular pathogens that require both CD4 T_H1 cells and CD8 T cells to clear them. Chronic infection is associated with marked pathology caused by the overproduction of IFN-γ and TNF-α by T cells and macrophages, respectively.

12-20 The mucosal immune system has to compromise between suppression and activation of an immune response.

The immune system in the normal intestine and other mucosal surfaces is biased to avoid making active immune responses against the majority of antigens encountered (see Section 12-15). But the antigens are still recognized, and potent protective immune responses must be, and are, generated against pathogens when required, as we saw in preceding sections. How can these apparently opposing needs be met without compromising the health of the host? The answer seems to be in the interactions between local dendritic cells and factors in the mucosal microenvironment (see Fig. 12.25).

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These interactions normally result in a population of noninflammatory dendritic cells, as discussed earlier in this chapter (see Section 12-8). Fortunately for our health, however, this predominantly inhibitory microenvironment can be changed by the presence of invasive pathogens or of adjuvants, allowing dendritic cells to be fully activated and productive immunity to be induced when required (see Fig. 12.25). The ability of mucosal dendritic cells to change their behavior rapidly and with high sensitivity probably reflects the fact that, even in the absence of overt infection, both the inflammatory and regulatory components of the immune response are likely to be operating simultaneously in the mucosa. The term physiological inflammation is used to describe the appearance of the normal intestine, which contains large numbers of lymphocytes and other cells that are normally associated with chronic inflammation and are generally not present in other organs in the absence of disease. This 'inflammation' is driven mainly by the presence of commensal bacteria and to a lesser extent by food antigens, and it is essential for the normal function of both the intestine and the mucosal immune system. It probably also ensures that dendritic cells are always in a state of high readiness to respond appropriately to changes in their local environment.

In addition to combating infection, these regulatory interactions may have had a wider influence on the evolution of the gut and the immune system, being one of the factors underlying the **hygiene hypothesis** (see Section 14-4). According to this idea, the human immune system has evolved in the face of continued exposure to ubiquitous pathogens and commensals, whose immunomodulatory products have helped to condition the polarization of responses to other foreign antigens. With the increasing cleanliness of the human environment, our immune system is no longer exposed to this influence during the critical early period of life, allowing hypersensitivity reactions of all kinds to develop unchecked against self antigens and harmless environmental substances. As the main source of exposure to environmental microbes, the intestine is heavily involved in these processes. In particular, there is clear evidence that the increasing incidence of disorders such as type 1 diabetes, Crohn's disease, and atopy correlates specifically with the eradication of immunomodulatory organisms such as helminths and *H. pylori* from the Western world.

Summary.

The immune system in the mucosa has to distinguish between potential pathogens and harmless antigens, generating strong effector responses to pathogens but remaining unresponsive to foods and commensals. Pathogenic microorganisms such as enteric bacteria use several strategies to invade, often exploiting the host's antigen-uptake and inflammatory mechanisms as well as modulating different components of the immune response. The strong T_H1 or T_H17 reactions they provoke normally result in elimination of the infection. In contrast, food proteins induce an active form of immunological tolerance in the systemic and mucosal immune systems, which may be mediated by regulatory T cells producing IL-10 and/or TGF-β. Commensal bacteria are also recognized by the immune system, but this is limited to the mucosa and its draining lymphoid tissues, because they are presented to T cells by dendritic cells that migrate from the intestinal wall and lodge in the draining mesenteric lymph node. This results in active mucosal tolerance and the production of local IgA antibodies that restrict colonization by the microorganisms, but 'ignorance' of these antigens by the systemic immune system. Because commensal bacteria have many beneficial effects for the host, these immunoregulatory processes are important in allowing the bacteria to coexist with the immune system.

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Intestinal helminths frequently produce chronic infections, partly because they produce several factors that can modulate the host immune system. The dominant protective response against helminths is T_H2 mediated, with the involvement of IgE, mast cells, and eosinophils and the production of TNF-α. Such a response can also damage the intestine, and the immune system maintains a balance between protective immunity and immunopathology. A similar situation occurs with *H. pylori* infection of the stomach, where this bacterium manipulates the host response to ensure the presence of a chronic inflammatory state that promotes its growth and prevents protective immunity. The absence of helminth- and *Helicobacter*-derived immunomodulatory factors may contribute to the increased incidence of allergic and inflammatory diseases in developed countries.

The key factor deciding between the generation of protective immunity and immune tolerance in the gut mucosa is local dendritic cells. Normally, intestinal dendritic cells do not respond to pro-inflammatory stimuli and will polarize the T-cell response toward the differentiation of gut-homing regulatory T cells. Nevertheless, the dendritic cells can still respond fully to invading organisms and inflammatory signals when required, allowing the priming of T cells to effector status. When the normal regulatory processes break down, inflammatory diseases can occur. As a consequence of these competing, but interacting, needs of the immune response, the intestine normally has the appearance of physiological inflammation, which helps maintain normal function of the gut and immune system.

Summary to Chapter 12.

The mucosal immune system is a large and complex apparatus that has a crucial role in health, not just by protecting physiologically vital organs but also by helping to regulate the tone of the entire immune system and prevent disease. The peripheral lymphoid organs focused on by most immunologists may be a recent specialization of an original template that evolved in mucosal tissues. The mucosal surfaces of the body are highly vulnerable to infection and possess a complex array of innate and adaptive mechanisms of immunity. The adaptive immune system of the mucosa-associated lymphoid tissues differs from that of the rest of the peripheral lymphoid system in several respects: the immediate juxtaposition of mucosal epithelium and lymphoid tissue; diffuse lymphoid tissue as well as more organized lymphoid organs; specialized antigen uptake mechanisms and dendritic cells; the predominance of activated/memory lymphocytes even in the absence of infection; the production of dimeric secretory IgA as the predominant antibody; and the downregulation of immune responses to innocuous antigens such as food antigens and commensal microorganisms. No systemic immune response can normally be detected to these antigens. In contrast, pathogenic microorganisms induce strong protective responses. The key factor in the decision between tolerance and the development of powerful adaptive immune responses is the context in which antigen is presented to T lymphocytes in the mucosal immune system. When there is no inflammation, presentation of antigen to T cells by specialized CD103-positive dendritic cells induces the differentiation of regulatory T cells. By contrast, pathogenic microorganisms crossing the mucosa induce an inflammatory response in the tissues, which stimulates the maturation of antigen-presenting cells and their expression of co-stimulatory molecules, thus favoring a protective T-cell response.

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Questions.

12.1 *Describe the processes that allow a specific CD4 T cell to be primed against antigen in the intestine, and discuss how the resulting effector T cells can return to the intestinal surface.*

12.2 *We are exposed to foreign antigens in large quantities in the food that we eat. (a) Why do we not mount effective immune responses against these food antigens? (b) How does the immune system distinguish between food antigens and antigens that are potentially harmful?*

12.3 *Discuss how dendritic cells in the intestine are distinct from their counterparts in other tissues, and describe how their functions are adapted to the local challenges faced by the immune system in the gut.*

12.4 *Describe how different aspects of the host immune response may produce either protective immunity or tissue damage during infection by an intestinal worm.*

12.5 *An unusual aspect of the intestine is the close interaction between epithelial cells and cells of the immune system. Outline how these interactions help shape immune responses to the different kinds of antigen encountered by the gut.*

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Autoimmunity and Transplantation

15

We have already seen how undesirable adaptive immune responses can be elicited by environmental antigens, and how this can cause serious disease in the form of allergic and hypersensitivity reactions (discussed in Chapter 14). In this chapter we examine unwanted responses to other medically important categories of antigens—those expressed on the body's own cells and tissues, on transplanted organs, or on the commensal microbiota that populate the intestinal tract. The responses to self antigens or antigens associated with the commensal microbiota are called **autoimmunity** and can lead to **autoimmune diseases** that are characterized by tissue damage. The response to nonself antigens on transplanted organs is called **allograft rejection**.

The gene rearrangements that occur during lymphocyte development in the central lymphoid organs are random, and thus inevitably result in the generation of some lymphocytes with affinity for self antigens. Such lymphocytes are normally removed from the repertoire or held in check by a variety of mechanisms, many of which we have already encountered in Chapter 8. These generate a state of **self-tolerance** in which an individual's immune system does not attack the normal tissues of the body. Autoimmunity represents a breakdown or failure of the mechanisms of self-tolerance. We therefore first revisit the mechanisms that keep the lymphocyte repertoire self-tolerant and see how these may fail. We then discuss a selection of autoimmune diseases that illustrate the various pathogenic mechanisms by which autoimmunity can damage the body. How genetic and environmental factors predispose to or trigger autoimmunity are then considered. In the remaining part of the chapter, we discuss the adaptive immune responses to nonself tissue antigens that cause transplant rejection.

The making and breaking of self-tolerance.

To generate self-tolerance, the immune system must be able to distinguish self-reactive from nonself-reactive lymphocytes as they develop. As we learned in Chapter 8, the immune system takes advantage of surrogate markers of self and nonself to identify and delete potentially self-reactive lymphocytes. Despite this, some self-reactive lymphocytes escape elimination, leave the thymus, and can subsequently be activated to cause autoimmune disease. In part, autoreactivity occurs because the recognition of self-reactivity is indirect and therefore imperfect. In addition, many lymphocytes with some degree of self-reactivity can also make an immune response to foreign antigens; therefore, if all weakly self-reactive lymphocytes were eliminated, the function of the immune system would be impaired.

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15-1 A critical function of the immune system is to discriminate self from nonself.

The immune system has very powerful effector mechanisms that can eliminate a wide variety of pathogens. Early in the study of immunity it was realized that these could, if turned against the host, cause severe tissue damage. The concept of autoimmunity was first presented at the beginning of the 20th century by **Paul Ehrlich**, who described it as '*horror autotoxicus*.' Autoimmune responses resemble normal immune responses to pathogens in that they are specifically activated by antigens, in this case self antigens or **autoantigens**, and give rise to autoreactive effector cells and to antibodies, called **autoantibodies**, against the self antigen. When reactions to self tissues do occur and are then improperly regulated, they cause a variety of chronic syndromes called autoimmune diseases. These syndromes are quite varied in their severity, in the tissues affected, and in the effector mechanisms that are most important in causing damage (Fig. 15.1).

Although individual autoimmune diseases are uncommon, collectively they affect approximately 5% of the populations of Western countries, and their incidence is on the rise. Nevertheless, their relative rarity indicates that the immune system has evolved multiple mechanisms to prevent damage to self tissues. The most fundamental principle underlying these mechanisms is the discrimination of self from nonself, but this discrimination is not easy to achieve. B cells recognize the three-dimensional shape of an epitope on an antigen, and a pathogen epitope can be indistinguishable from one in humans. Similarly, the short peptides derived from the processing of pathogen antigens can be identical to self peptides. So how does a lymphocyte know what 'self' really is if there are no unique molecular signatures of self?

Fig. 15.1 Some common autoimmune diseases. The diseases listed are among the commonest autoimmune diseases and will be used as examples in this part of the chapter. They are listed in order of prevalence. A more comprehensive listing and discussion of autoimmune diseases is given later in the chapter.

Disease	Disease mechanism	Consequence	Prevalence
Psoriasis	Autoreactive T cells against skin-associated antigens	Inflammation of skin with formation of scaly patches or plaques	1 in 50
Rheumatoid arthritis	Autoreactive T cells against antigens of joint synovium	Joint inflammation and destruction causing arthritis	1 in 100
Graves' disease	Autoantibodies against the thyroid-stimulating-hormone receptor	Hyperthyroidism: overproduction of thyroid hormones	1 in 100
Hashimoto's thyroiditis	Autoantibodies and autoreactive T cells against thyroid antigens	Destruction of thyroid tissue leading to hypothyroidism: underproduction of thyroid hormones	1 in 200
Systemic lupus erythematosus	Autoantibodies and autoreactive T cells against DNA, chromatin proteins, and ubiquitous ribonucleoprotein antigens	Glomerulonephritis, vasculitis, rash	1 in 200
Sjögren's syndrome	Autoantibodies and autoreactive T cells against ribonucleoprotein antigens	Lymphocyte infiltration of exocrine glands, leading to dry eyes and/or dry mouth; other organs may be involved, leading to systemic disease	1 in 300
Crohn's disease	Autoreactive T cells against intestinal flora antigens	Intestinal inflammation and scarring	1 in 500
Multiple sclerosis	Autoreactive T cells against brain antigens	Formation of sclerotic plaques in brain with destruction of myelin sheaths surrounding nerve cell axons, leading to muscle weakness, ataxia, and other symptoms	1 in 700
Type 1 diabetes (insulin-dependent diabetes mellitus, IDDM)	Autoreactive T cells against pancreatic islet cell antigens	Destruction of pancreatic islet β cells leading to nonproduction of insulin	1 in 800

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The first mechanism proposed for distinguishing between self and nonself was that recognition of antigen by an immature lymphocyte leads to a negative signal that causes lymphocyte death or inactivation. Thus, 'self' was thought to comprise those molecules recognized by a lymphocyte shortly after it has begun to express its antigen receptor. Indeed, this is an important mechanism of inducing self-tolerance in lymphocytes developing in the thymus and bone marrow. The tolerance induced at this stage is known as **central tolerance** and is discussed in detail in Chapter 8. Newly formed lymphocytes are especially sensitive to inactivation by strong signals through their antigen receptors, whereas the same signals would activate a mature lymphocyte.

Another antigenic quality that correlates with self is a sustained, high concentration of the antigen. Many self proteins are expressed by multiple cell types in the body or are abundant in connective tissues. These can provide strong signals to lymphocytes, and even mature lymphocytes can be made tolerant to an antigen, or **tolerized**, by strong and constant signals through their antigen receptors. In contrast, pathogens and other foreign antigens are introduced to the immune system suddenly, and the concentrations of their antigens increase rapidly and exponentially as the pathogens replicate in the early stages of an infection. Naive mature lymphocytes are tuned to respond by activation to a sudden increase in antigen-receptor signals.

A third mechanism for discriminating between self and nonself relies on the innate immune system, which provides signals that are crucial in enabling the activation of an adaptive immune response to infection (see Chapter 3). In the absence of infection, these signals, which include pro-inflammatory cytokines (for example IL-6 or IL-12) and co-stimulatory molecules (for example B7.1) that are expressed by activated antigen-presenting cells, are not generated. In these circumstances, the encounter of a naive lymphocyte with a self antigen tends to lead to a negative inactivating signal, rather than no signal at all (see Section 8-26), or can promote the development of regulatory lymphocytes that suppress the development of effector responses that might injure tissues. This tolerance mechanism is particularly important for antigens that are encountered outside the thymus and bone marrow. Tolerance induced in the mature lymphocyte repertoire once cells have left the central lymphoid organs is known as **peripheral tolerance**.

Thus, several clues are used by lymphocytes to distinguish self ligands from nonself ligands: encounter with the ligand when the lymphocyte is still immature; a high and constant concentration of ligand; and binding the ligand in the absence of pro-inflammatory cytokine or co-stimulatory signals. All these mechanisms are error-prone, however, because none of them particularly distinguishes a self ligand from a foreign one at the molecular level. The immune system therefore has several additional mechanisms for controlling autoimmune responses should they start.

15-2 Multiple tolerance mechanisms normally prevent autoimmunity.

The mechanisms that normally prevent autoimmunity may be considered as a succession of checkpoints. Each checkpoint is partly effective in preventing anti-self responses, and together they act synergistically to provide efficient protection against autoimmunity without inhibiting the ability of the immune system to mount effective responses to pathogens. Central tolerance mechanisms eliminate newly formed strongly autoreactive lymphocytes. On the other hand, mature self-reactive lymphocytes that do not sense self strongly in the central lymphoid organs—because their cognate self antigens are not expressed there, for example—may be killed or inactivated in the periphery. The principal mechanisms of peripheral tolerance are anergy (functional unresponsiveness), suppression by regulatory T cells, induction of regulatory T-cell development instead of effector T-cell development

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(functional deviation), and deletion of lymphocytes from the repertoire due to activation-induced cell death. In addition, some antigens are sequestered in organs that are not normally accessible to the immune system (Fig. 15.2).

Each checkpoint strikes a balance between preventing autoimmunity and not impairing immunity too greatly, and in combination they provide an effective overall defense against autoimmune disease. It is relatively easy to find isolated breakdowns of one or even more layers of protection, even in healthy individuals. Thus, activation of autoreactive lymphocytes does not necessarily equal autoimmune disease. In fact, a low level of autoreactivity is physiological and crucial to normal immune function. Autoantigens help to form the repertoire of mature lymphocytes, and the survival of naive T cells and B cells in the periphery requires continuous exposure to autoantigens (see Chapter 8). Autoimmune disease develops only if enough of the safeguards are overcome to lead to a sustained reaction to self that includes the generation of effector cells and molecules that destroy tissues. Although the mechanisms by which this occurs are not completely known, autoimmunity is thought to result from a combination of genetic susceptibility, breakdown in natural tolerance mechanisms, and environmental triggers such as infections (Fig. 15.3).

15-3 Central deletion or inactivation of newly formed lymphocytes is the first checkpoint of self-tolerance.

Central tolerance mechanisms, which remove strongly autoreactive lymphocytes, are the first and most important checkpoints in self-tolerance and are covered in detail in Chapter 8. Without them, the immune system would be strongly self-reactive, and lethal autoimmunity would most certainly be present from birth. It is unlikely that the other, later-acting, mechanisms of tolerance would be sufficient to compensate for the failure to remove self-reactive lymphocytes during their primary development. Indeed, there are

Fig. 15.2 Self-tolerance depends on the concerted action of a variety of mechanisms that operate at different sites and stages of development. The different ways in which the immune system prevents activation of and damage caused by autoreactive lymphocytes are listed, along with the specific mechanism and where such tolerance predominantly occurs.

Layers of self-tolerance		
Type of tolerance	**Mechanism**	**Site of action**
Central tolerance	Deletion Editing	Thymus Bone marrow
Antigen segregation	Physical barrier to self-antigen access to lymphoid system	Peripheral organs (e.g. thyroid, pancreas)
Peripheral anergy	Cellular inactivation by weak signaling without co-stimulus	Secondary lymphoid tissue
Regulatory T cells	Suppression by cytokines, intercellular signals	Secondary lymphoid tissue and sites of inflammation
Functional deviation	Differentiation of regulatory T cells that limit inflammatory cytokine secretion	Secondary lymphoid tissue and sites of inflammation
Activation-induced cell death	Apoptosis	Secondary lymphoid tissue and sites of inflammation

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no known autoimmune diseases that are attributable to a complete failure of these basic mechanisms, although some are associated with a partial failure of central tolerance.

Self-tolerance generated in the central lymphoid organs is effective, but for a long time it was thought that many self antigens were not expressed in the thymus or bone marrow, and that peripheral mechanisms must be the only way of generating tolerance to them. It is now clear, however, that many (but not all) tissue-specific antigens, such as insulin, are actually expressed in the thymus by a subset of dendritic-like cells, and thus self-tolerance against these antigens can be generated centrally. How these 'peripheral' genes are turned on ectopically in the thymus is not yet completely worked out, but an important clue has been found. A single transcription factor, AIRE (for *a*uto*i*mmune *re*gulator), is thought to be responsible for turning on many peripheral genes in the thymus (see Section 8-20). The *AIRE* gene is defective in patients with a rare inherited form of autoimmunity—**APECED** (**autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy**)—that leads to the destruction of multiple endocrine tissues, including insulin-producing pancreatic islets. This disease is also known as autoimmune polyglandular syndrome 1 (APS-1). Mice that have been engineered to lack the *AIRE* gene have a similar syndrome, although they do not seem to be susceptible to fungal infections such as candidiasis. Most importantly, these mice no longer express many of the peripheral genes in the thymus. This links the AIRE protein to the expression of these genes as well as suggesting that an inability to express these genes in the thymus leads to autoimmune disease (Fig. 15.4). The autoimmunity that accompanies AIRE deficiency takes time to develop and does not always affect all potential organ targets. So as well as emphasizing the importance of central tolerance, the disease shows that other layers of tolerance control have important roles.

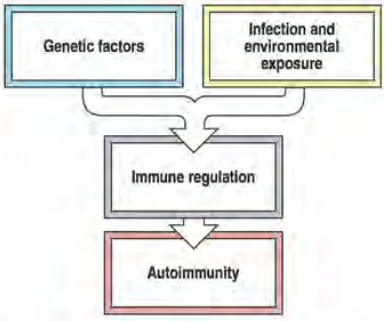


Fig. 15.3 Requirements for the development of autoimmune disease. In genetically predisposed individuals, autoimmunity may be triggered as a result of the failure of intrinsic tolerance mechanisms and/or environmental triggers such as infection.



Autoimmune Polyendocrinopathy–Candidiasis–Ectodermal Dystrophy (APECED)

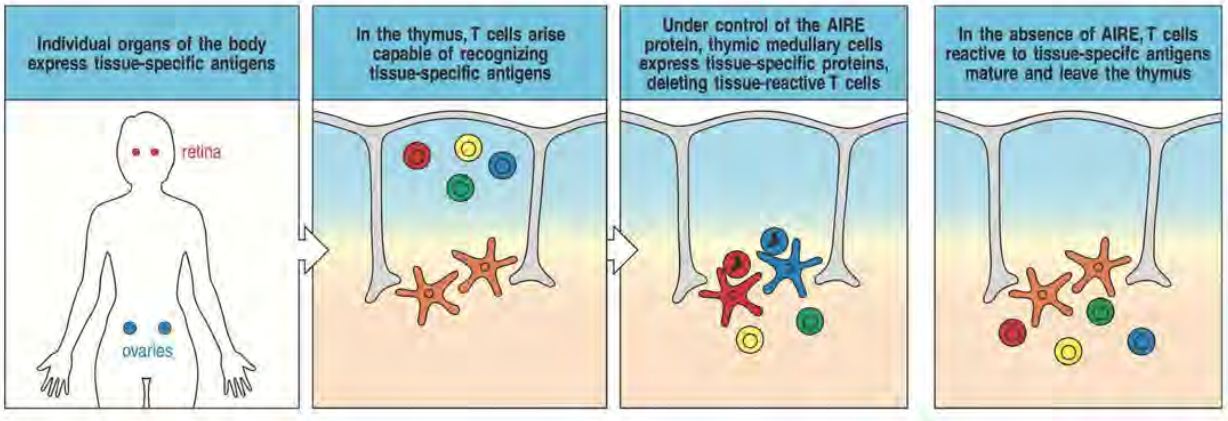


Fig. 15.4 The 'autoimmune regulator' gene *AIRE* promotes the expression of some tissue-specific antigens in thymic medullary cells, causing the deletion of immature thymocytes that can react to these antigens. Although the thymus expresses many genes, and thus self proteins, common to all cells, it is not obvious how antigens that are specific to specialized tissues, such as retina or ovary (first panel), gain access to the thymus to promote the negative selection of immature autoreactive thymocytes. It is now known that a gene called *AIRE* promotes the expression of many tissue-specific proteins in thymic medullary cells. Some developing thymocytes will be able to recognize these tissue-specific antigens (second panel). Peptides from these proteins are presented to the developing thymocytes as they undergo negative selection in the thymus (third panel), causing deletion of these cells. In the absence of *AIRE*, this deletion does not occur; instead, the autoreactive thymocytes mature and are exported to the periphery (fourth panel), where they could cause autoimmune disease. Indeed, people and mice that lack expression of *AIRE* develop an autoimmune syndrome called APECED, or autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy.

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15-4 Lymphocytes that bind self antigens with relatively low affinity usually ignore them but in some circumstances become activated.

Most circulating lymphocytes have a low affinity for self antigens but make no response to them, and may be considered as 'ignorant' of self (see Section 8-6). Such ignorant but latently self-reactive cells can be recruited into autoimmune responses if their threshold for activation is lowered by co-activating factors. One such stimulus could be infection. Naive T cells with low affinity for a ubiquitous self antigen can become activated if they encounter an activated dendritic cell presenting that antigen and expressing high levels of co-stimulatory signals or pro-inflammatory cytokines as a result of the presence of infection.

A particular situation in which ignorant lymphocytes may be activated is where their autoantigens are also the ligands for Toll-like receptors (TLRs). These receptors are usually considered to be pattern-recognition receptors specific for pathogen-associated molecular patterns (see Section 3-7), but these patterns are not exclusive to pathogens and can be found among self molecules. An example of this type of potential autoantigen is unmethylated CpG sequences in DNA that are recognized by TLR-9. Unmethylated CpG is normally much more common in bacterial DNA than in mammalian DNA but is enriched in mammalian cells undergoing apoptosis. In a scenario of extensive cell death coupled with inadequate clearance of apoptotic fragments (possibly as a result of infection), B cells specific for components of chromatin can internalize the CpG sequences via their B-cell receptors. These sequences encounter their receptor, TLR-9, intracellularly, leading to a co-stimulatory signal that, together with the signal from the B-cell receptor, activates the previously ignorant anti-chromatin B cell (Fig. 15.5). B cells activated in this way will proceed to produce anti-chromatin autoantibodies and also can act as antigen-presenting cells for autoreactive T cells. Ribonucleoprotein complexes containing uridine-rich RNA have similarly been shown to activate naive B cells through binding of the RNA by TLR-7 or TLR-8. Autoantibodies against DNA, chromatin proteins, and ribonucleoproteins are produced in the autoimmune disease **systemic lupus erythematosus** (**SLE**), and this may be one of the mechanisms by which self-reactive B cells are stimulated to produce them. These findings challenge the concept that TLRs are completely reliable at distinguishing self from nonself; their proposed role in autoimmunity has been called the 'Toll hypothesis.'

Another mechanism by which ignorant lymphocytes can be drawn into action is by changing the availability or form of a self antigen. Some antigens are normally intracellular and are not encountered by lymphocytes, but they may be released as a result of massive tissue death or inflammation. These antigens

Systemic Lupus Erythematosus

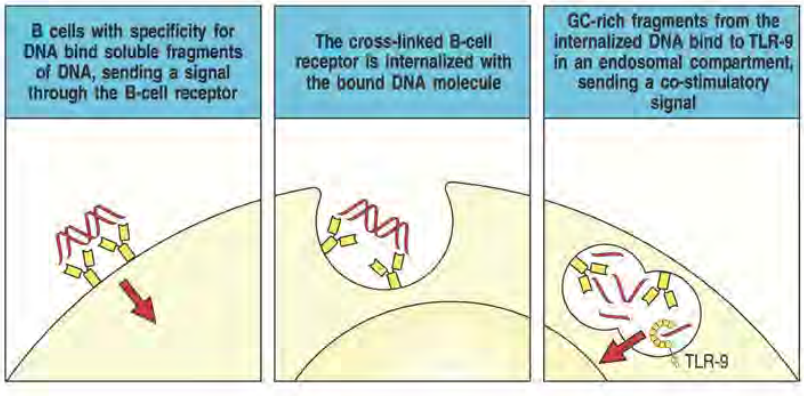


Fig. 15.5 Self antigens that are recognized by Toll-like receptors can activate autoreactive B cells by providing co-stimulation. The receptor TLR-9 promotes the activation of B cells specific for DNA, a common autoantibody in the autoimmune disease systemic lupus erythematosus (SLE) (see Fig. 15.1). Although B cells with strong affinity for DNA are eliminated in the bone marrow, some DNA-specific B cells with lower affinity escape and persist in the periphery but are not normally activated. Under some conditions and in genetically susceptible individuals, the concentration of DNA may increase, leading to the ligation of enough B-cell receptors to initiate activation of these B cells. B cells signal through their receptor (left panel) but also take up the DNA (center panel) and deliver it to an endosomal compartment (right panel). Here the DNA has access to TLR-9, which recognizes DNA that is enriched in unmethylated CpG DNA sequences. Such CpG-enriched sequences are much more common in microbial than eukaryotic DNA and normally this allows TLR-9 to distinguish pathogens from self. DNA in apoptotic mammalian cells is enriched in unmethylated CpG, however, and the DNA-specific B cell will also concentrate the self DNA in the endosomal compartment. This would provide sufficient ligand to activate TLR-9, potentiating the activation of the DNA-specific B cell and leading to the production of autoantibodies against DNA.

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can then activate hitherto ignorant T and B cells, leading to autoimmunity. This can occur after myocardial infarction, when an autoimmune response is detectable some days after the release of cardiac antigens. Such reactions are typically transient and cease when the autoantigens have been removed; however, when clearance mechanisms are inadequate or genetically deficient, they can continue, causing clinical autoimmune disease.

Some autoantigens are present in great quantity but are usually in a nonimmunogenic form. IgG is a good example, because there are large quantities of it in blood and in other extracellular fluids. B cells specific for the IgG constant region are not usually activated because the IgG is monomeric and cannot cross-link the B-cell receptor. However, when immune complexes form after a severe infection or an immunization, enough IgG is in multivalent form to evoke a response from these otherwise ignorant B cells. The anti-IgG autoantibody they produce is known as **rheumatoid factor** because it is commonly present in rheumatoid arthritis. Again, this response is normally short lived, as long as the immune complexes are cleared rapidly.

A unique situation occurs in peripheral lymphoid organs when activated B cells undergo somatic hypermutation in germinal centers (see Section 10-7). This can result in some already activated B cells becoming self-reactive or increasing their affinity for a self antigen (Fig. 15.6). Like the ignorant lymphocytes discussed above, such self-reactive B cells would have bypassed all the other tolerance mechanisms but would now be a source of potentially pathogenic autoantibodies. There seems, however, to be a mechanism to control germinal-center B cells that have acquired affinity for self. In this case, the self antigen is likely to be present within the germinal center, whereas a pathogen is less likely to be. If a hypermutated self-reactive B cell encounters strong cross-linking of its B-cell receptor in the germinal center, it undergoes apoptosis rather than further proliferation.

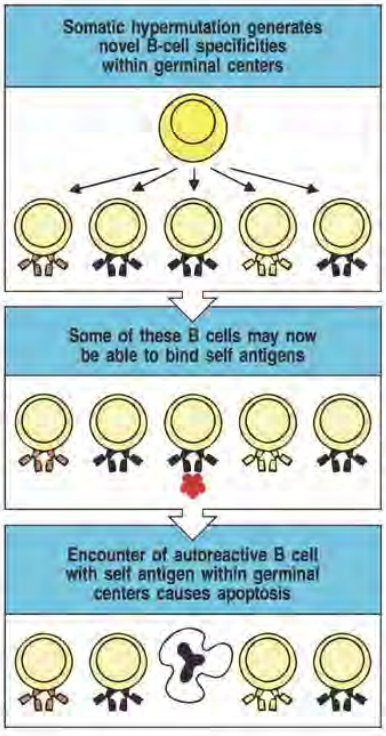


Fig. 15.6 Elimination of autoreactive B cells in germinal centers. During somatic hypermutation in germinal centers (top panel), B cells with autoreactive B-cell receptors can arise. Ligation of these receptors by soluble autoantigen (center panel) induces apoptosis of the autoreactive B cell by signaling through the B-cell antigen receptor in the absence of helper T cells (bottom panel).

15-5 Antigens in immunologically privileged sites do not induce immune attack but can serve as targets.

Tissue grafts placed in some sites in the body do not elicit immune responses. For instance, the brain and the anterior chamber of the eye are sites in which tissues can be grafted without inducing rejection. Such locations are termed **immunologically privileged sites** (Fig. 15.7). It was originally believed that immunological privilege arose from the failure of antigens to leave privileged sites and induce immune responses. Subsequent studies have shown that antigens do leave these sites and that they do interact with T cells. Instead of eliciting a destructive immune response, however, they induce tolerance or a response that is not destructive to the tissue.

Immunologically privileged sites seem to be unusual in three ways. First, the communication between the privileged site and the body is atypical in that extracellular fluid in these sites does not pass through conventional lymphatics, although proteins placed in these sites do leave them and can have immunological effects. Privileged sites are generally surrounded by tissue barriers that exclude naive lymphocytes. The brain, for example, is guarded by the blood–brain barrier. Second, soluble factors, presumably cytokines, that affect the course of an immune response are produced in privileged sites and leave them together with antigens. The anti-inflammatory transforming growth factor (TGF)-β seems to be particularly important in this regard. In

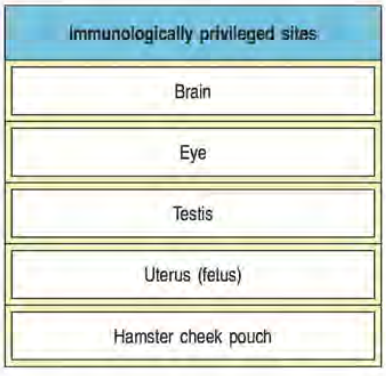


Fig. 15.7 Some sites in the body are immunologically privileged. Tissue grafts placed in these sites often last indefinitely, and antigens placed in these sites do not elicit destructive immune responses.

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homeostatic conditions (that is, in the absence of infection and thus of pro-inflammatory signals), antigens recognized in concert with TGF-β tend to induce regulatory T-cell responses that do not damage tissues, rather than pro-inflammatory T_H17 responses, which are induced by TGF-β in the presence of IL-6 co-signaling (see Section 9-18). Third, the expression of Fas ligand by the tissues of immunologically privileged sites may provide a further level of protection by inducing the apoptosis of Fas-bearing effector lymphocytes that enter these sites.

Paradoxically, the antigens sequestered in immunologically privileged sites are often the targets of autoimmune attack; for example, brain autoantigens such as myelin basic protein are targeted in the autoimmune disease **multiple sclerosis**, a chronic inflammatory demyelinating disease of the central nervous system (see Fig. 15.1). It is therefore clear that the tolerance normally shown to this antigen cannot be due to previous deletion of the self-reactive T cells. In the condition **experimental autoimmune encephalomyelitis** (**EAE**), a mouse model for multiple sclerosis, mice become diseased only when they are deliberately immunized with myelin basic protein, which causes substantial infiltration of the brain with antigen-specific T_H17 and T_H1 cells that cooperate to induce a local inflammatory response that damages nerve tissue.

This shows that at least some antigens expressed in immunologically privileged sites induce neither tolerance nor lymphocyte activation in normal circumstances, but if autoreactive lymphocytes are activated elsewhere, these autoantigens can become targets for autoimmune attack. It seems plausible that T cells specific for antigens sequestered in immunologically privileged sites are most likely to be in a state of immunological ignorance. Further evidence comes from the eye disease **sympathetic ophthalmia** (Fig. 15.8). If one eye is ruptured by a blow or other trauma, an autoimmune response to eye proteins can occur, although this happens only rarely. Once the response is induced, it often attacks both eyes. Immunosuppression—and, rarely, removal of the damaged eye, the source of antigen—is required to preserve vision in the undamaged eye.

It is not surprising that effector T cells can enter immunologically privileged sites: such sites can become infected, and effector cells must be able to enter these sites during infection. Effector T cells enter most or all tissues after

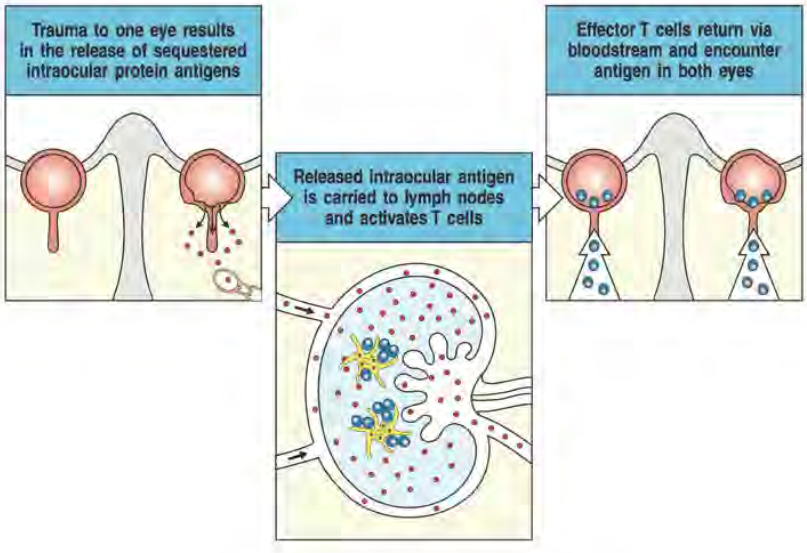


Fig. 15.8 Damage to an immunologically privileged site can induce an autoimmune response. In the disease sympathetic ophthalmia, trauma to one eye releases the sequestered eye antigens into the surrounding tissues, making them accessible to T cells. The effector cells that are elicited attack the traumatized eye, and also infiltrate and attack the healthy eye. Thus, although the sequestered antigens do not induce a response by themselves, if a response is induced elsewhere they can serve as targets for attack.

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activation (see Chapter 11), but accumulation of cells is seen only when antigen is recognized in the site, triggering the production of cytokines that alter tissue barriers.

15-6 Autoreactive T cells that express particular cytokines may be nonpathogenic or may suppress pathogenic lymphocytes.

We learned in Chapter 9 that, during the course of normal immune responses, CD4 T cells can differentiate into various types of effector cells, namely T_H1, T_H2, and T_H17. T_H1, T_H2, and T_H17 cells secrete different cytokines (interferon (IFN)-γ and tumor necrosis factor (TNF)-α for T_H1; interleukin (IL)-4, IL-5, IL-10, and IL-13 for T_H2; and IL-17 and IL-22 for T_H17). These effector cell subsets probably evolved to control different types of infectious threats and therefore orchestrate distinct types of immunity, which is reflected in their different effects on antigen-presenting cells, B cells, and innate effector cells such as macrophages, eosinophils, and neutrophils. A similar paradigm holds true for autoimmunity. In particular, certain T cell-mediated autoimmune diseases such as **type 1 diabetes mellitus** (also known as **insulin-dependent diabetes mellitus** or **IDDM**) (see Fig. 15.1) depend on T_H1 cells to cause disease, whereas others, such as psoriasis (an autoimmune disease of the skin), depend on T_H17 cells.

In murine models of diabetes, when cytokines were infused to influence T-cell differentiation or when knockout mice predisposed to T_H2 differentiation were studied, the development of diabetes was inhibited. In some cases, potentially pathogenic T cells specific for pancreatic islet-cell components, and expressing T_H2 instead of T_H1 cytokines, are actually suppressive of disease caused by T_H1 cells of the same specificity. So far, attempts to control human autoimmune disease by switching cytokine profiles from one effector cell type to another (for example T_H1 to T_H2), a procedure termed **immune modulation**, have not proved successful. Another important subset of CD4 T cells, the regulatory T cells, may prove to be more important in the natural prevention of autoimmune disease, and efforts to deviate effector T-cell responses to regulatory T-cell responses by immune modulation may have promise as a novel therapy for treatment of autoimmunity.

15-7 Autoimmune responses can be controlled at various stages by regulatory T cells.

Autoreactive cells that have escaped the tolerance-inducing mechanisms described previously can still be regulated so that they do not cause clinical disease. This regulation takes two forms: the first is extrinsic, coming from specific regulatory T cells that exert effects on activated T cells and on antigen-presenting cells. The second is intrinsic and has to do with limits on the size and duration of immune responses that are programmed into the lymphocytes themselves. We shall first discuss the role of regulatory T cells, which were introduced in Chapter 9.

Tolerance due to regulatory lymphocytes is distinguished from other forms of self-tolerance by the fact that a regulatory T (T_{reg}) cell has the potential to suppress self-reactive lymphocytes that recognize antigens different from those recognized by the T_{reg} cell (Fig. 15.9). This type of tolerance is therefore known as **regulatory tolerance** or **infectious tolerance**. The key feature of regulatory tolerance is that regulatory cells can suppress autoreactive lymphocytes that recognize a variety of different self antigens, as long as the antigens are from the same tissue or are presented by the same antigen-presenting cell. Two general types of regulatory T cells have been defined experimentally. One type, referred to as 'natural' T_{reg} cells, are moderately autoreactive CD4 CD25 T cells expressing the transcription factor FoxP3 that escape deletion in the

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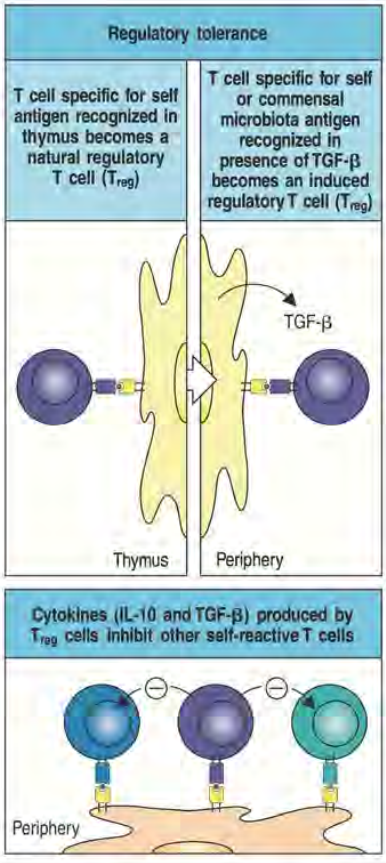


Fig. 15.9 Tolerance mediated by regulatory T cells can inhibit multiple autoreactive T cells that all recognize the same tissue. Specialized autoreactive natural regulatory T (T_{reg}) cells develop in the thymus in response to weak stimulation by self antigens that is not sufficient to cause deletion but is greater than that required for simple positive selection (upper left panel). Regulatory T cells can also be induced from naive self-reactive T cells in the periphery if the naive T cell recognizes its antigen and is activated in the presence of the cytokine TGF-β (upper right panel). The lower panel shows how regulatory T cells, both natural or induced, can inhibit other self-reactive T cells. If regulatory T cells encounter their self antigen on an antigen-presenting cell, they secrete inhibitory cytokines such as IL-10 and TGF-β that inhibit all surrounding autoreactive T cells, regardless of their precise autoantigen specificity.

thymus and when activated by self antigens in peripheral tissues do not differentiate into cells that can initiate an autoimmune response. Instead, they inhibit other self-reactive T cells that recognize antigens in the same tissue and prevent their differentiation into effector T cells or prevent their effector function. The second type, referred to as 'induced' or 'adaptive' T_{reg} cells, develop in peripheral immune tissues in response to antigens recognized on 'immature' dendritic cells that produce TGF-β in the absence of pro-inflammatory cytokines. Giving animals large amounts of self antigen orally, which induces so-called oral tolerance (see Section 12-15), can sometimes lead to unresponsiveness to these antigens when given by other routes, and can prevent autoimmune disease. Oral tolerance is routinely generated to antigens such as food antigens and is accompanied by the generation of induced T_{reg} cells in the gut-draining mesenteric lymph nodes. These cells are known to suppress immune responses to the given antigen in the gut itself, but how the suppression in the rest of the peripheral immune system is achieved is not yet known. Many investigators have hypothesized that T_{reg} cells could have therapeutic potential for the treatment of autoimmune disease if they could be isolated or induced to differentiate and then be infused into patients.

A common characteristic of all natural and many induced T_{reg} cells is the expression of CD4 and CD25 (the α chain of the IL-2 receptor) on their surface, and their expression of the transcription factor, FoxP3 (see Section 9-19). That FoxP3, and the T_{reg} cells whose development and function it controls, is important to the maintenance of immune tolerance is evident from the fact that humans and mice that carry mutations in the gene for FoxP3 rapidly develop severe, systemic autoimmunity (discussed in Section 15-20). A protective role for FoxP3-expressing T_{reg} cells has been demonstrated in respect of several autoimmune syndromes in mice, including diabetes, EAE, SLE, and inflammation of the large intestine, or colon (colitis). Experiments in mouse models of these diseases show that CD4 CD25 T_{reg} cells suppress disease when transferred *in vivo* and that depletion of these cells exacerbates or causes disease. A proposed model for the resolution of autoimmune colitis in mice by CD4 CD25 T_{reg} cells is shown in Fig. 15.10. These CD4 CD25 T_{reg} cells have also been shown to prevent or ameliorate other immunopathologic syndromes, such as graft-versus-host disease and graft rejection, which are described later in this chapter.

The importance of regulatory T cells has been demonstrated in several human autoimmune diseases. For example, in patients with multiple sclerosis or with autoimmune polyglandular syndrome type 2 (a rare syndrome in which two or more autoimmune diseases occur simultaneously), the suppressive activity of CD4 CD25 T_{reg} cells is defective, although their numbers are normal. A different picture emerges from studies of patients with active rheumatoid arthritis. Peripheral blood CD4 CD25 T_{reg} cells from these patients were found to be effective in suppressing the proliferation of the patients' own effector T cells *in vitro* but did not suppress the secretion of inflammatory cytokines by these cells. Thus, increasing evidence supports the notion that regulatory T cells normally have an important role in preventing autoimmunity, and that autoimmunity may be accompanied by a variety of functional defects in these cells.

FoxP3-expressing T_{reg} cells are not the only type of regulatory lymphocyte that has been found. Regulatory T cells that do not express FoxP3 have been identified *in vitro* and *in vivo*. These cells are characterized by their production of IL-10 and are enriched in the intestinal tissues, where they have been shown experimentally in mice to suppress inflammatory bowel disease (IBD), an autoinflammatory disease, through an IL-10-dependent mechanism.

Almost every type of lymphocyte has been shown to display regulatory activity in some circumstance. Even B cells can regulate experimentally induced

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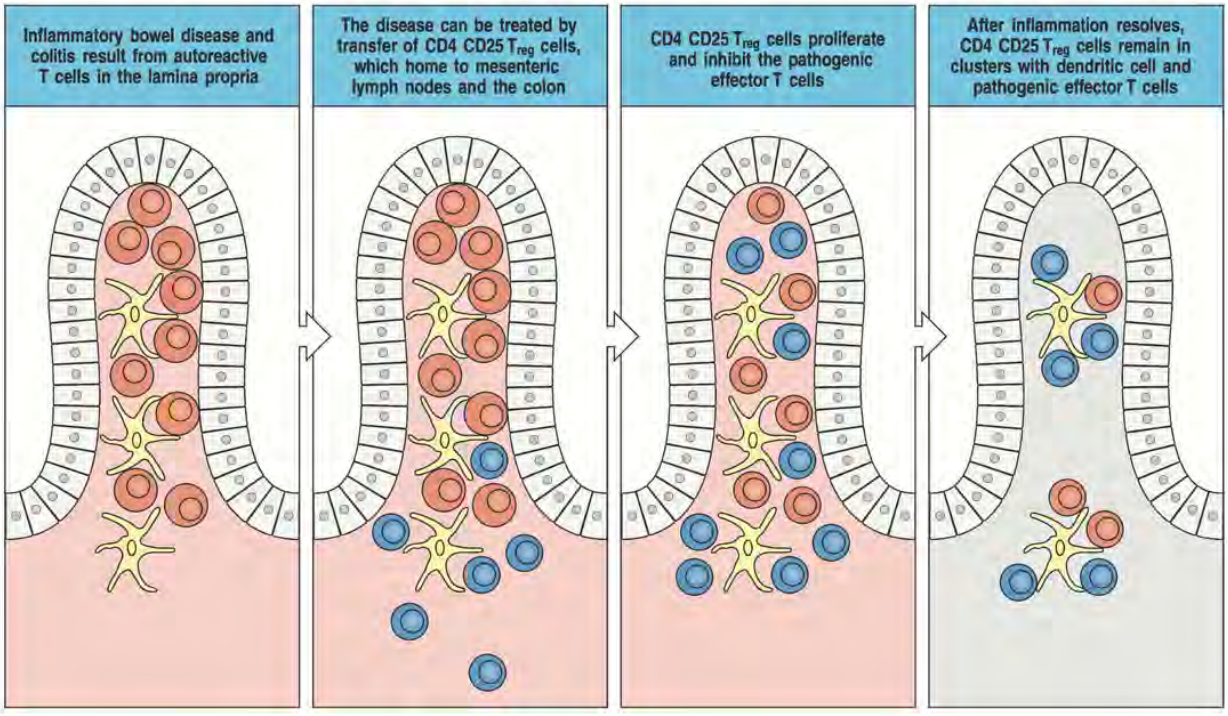


Fig. 15.10 CD4 CD25 regulatory T cells inhibit colitis by migrating to the colon and mesenteric lymph nodes, where they interact with dendritic and effector T cells. Naive T cells that contain some autoreactive clones (first panel, pink cells) cause colitis when transferred to T-cell-deficient mice. The naive population lacks CD4 CD25 T_{reg} cells, but if these are also transferred along with the naive T cells (second panel; blue cells are T_{reg} cells), colitis is blocked. The blocking mechanism includes migration of the T_{reg} cells to mesenteric lymph nodes (not shown) and later to the lamina propria of the colon. The T_{reg} cells proliferate and secrete regulatory cytokines (third panel), including IL-10, which is essential, and interact with both dendritic and autoreactive T cells, reducing activation (indicated by the smaller size of the pink cells) and ultimately reducing inflammation. Once inflammation has been quelled, regulatory T cells remain in the lamina propria (fourth panel). Based on a figure by F. Powrie.

autoimmune syndromes, including collagen-induced arthritis (CIA) and EAE in mice. This regulatory activity is probably mediated in a similar way to that of regulatory CD4 T cells, with the secretion of cytokines that inhibit T-cell proliferation and the differentiation of effector T cells being of major importance.

In addition to the extrinsic regulation of autoreactive T and B cells by regulatory cells, lymphocytes have intrinsic limits to proliferation and survival that can help to restrict autoimmune responses as well as normal immune responses (see Section 11-12). This is illustrated by the effects of mutations in the pathways that control apoptosis, such as the Bcl-2 pathway or the Fas pathway (see Section 7-23), that lead to spontaneous autoimmunity, as we shall see later in this chapter. This form of autoimmunity provides evidence that autoreactive cells are normally generated but are then controlled by apoptosis. This seems to be an important mechanism for both T- and B-cell tolerance.

Summary.

Discrimination between self and nonself is imperfect, partly because of its indirect nature and partly because a proper balance must be struck between preventing autoimmune disease and preserving immune competence. Self-reactive lymphocytes always exist in the natural immune repertoire but are not often activated. In autoimmune disease, however, these cells become activated by specific autoantigens. If activation persists, effector functions identical to those elicited in response to pathogens are generated and cause disease. The immune system has a remarkable set of mechanisms that work together to prevent autoimmune disease (see Fig. 15.2). This collective action means that each mechanism need not work perfectly nor apply to every possible self-reactive cell. Self-tolerance begins during lymphocyte development,

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when autoreactive T cells in the thymus and B cells in the bone marrow are deleted or, in the case of CD4 T cells, give rise to a subpopulation of self antigen-reactive, natural CD4 CD25 regulatory T (T_{reg}) cells that tend to suppress immune responses. Mechanisms of peripheral tolerance, such as peripheral anergy and deletion, or the extrathymic development of induced T_{reg} cells, complement these central tolerance mechanisms for antigens that are not expressed in the thymus or bone marrow. Weakly self-reactive lymphocytes are not removed at this stage; extending tolerance mechanisms such as deletion to weakly autoreactive cells would impose too great a limitation on the immune repertoire, resulting in impaired immune responses to pathogens. Instead, weakly self-reactive cells are suppressed only if they are activated, by mechanisms that include inhibition by T_{reg} cells, which are themselves autoreactive, although not pathogenic. Regulatory T cells can inhibit a variety of self-reactive lymphocytes, as long as the regulatory cells are targeting autoantigens located in the same general vicinity of the autoantigens to which the self-reactive lymphocytes respond. This allows the regulatory cells to home to and suppress sites of autoimmune inflammation. A final mechanism that controls autoimmunity is the natural tendency of immune responses to be self-limited: intrinsic programs in activated lymphocytes make them prone to apoptosis. Activated lymphocytes also acquire sensitivity to external apoptosis-inducing signals, such as those mediated by Fas.

Autoimmune diseases and pathogenic mechanisms.

Here we describe some of the more common clinical autoimmune syndromes, and the ways in which loss of self-tolerance and expansion of self-reactive lymphocytes lead to tissue damage. These mechanisms of pathogenesis resemble in many ways those that target invading pathogens. Damage by autoantibodies, mediated through the complement and Fc receptor systems, has an important role in some diseases, such as SLE. Similarly, cytotoxic T cells directed at self tissues destroy them much as they would virus-infected cells, and this is one way in which pancreatic β cells are destroyed in diabetes. However, self proteins cannot normally be eliminated, with rare exceptions such as those uniquely expressed by islet cells in the pancreas, and so the response continues. Some pathogenic mechanisms are unique to autoimmunity, such as antibodies against receptors on cell surfaces that affect their function, as in the disease myasthenia gravis, as well as hypersensitivity-type reactions. In this part of the chapter we describe the pathogenic mechanisms of some clinical syndromes of autoimmune disease.

15-8 Specific adaptive immune responses to self antigens can cause autoimmune disease.

In certain genetically susceptible strains of experimental animals, autoimmune disease can be induced artificially by the injection of 'self' tissues from a genetically identical animal that have been mixed with strong adjuvants containing bacteria (see Appendix I, Section A-4). This shows directly that autoimmunity can be provoked by inducing a specific adaptive immune response to self antigens. Such experimental systems highlight the importance of the activation of other components of the immune system, primarily dendritic cells, by the bacteria contained in the adjuvant. There are drawbacks to the use of such animal models for the study of autoimmunity, however. In humans and genetically autoimmune-prone animals, autoimmunity usually arises spontaneously: that is, we do not know what events initiate the immune response to self that leads to the autoimmune disease. By studying

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the patterns of autoantibodies and also the particular tissues affected, it has been possible to identify some of the self antigens that are targets of autoimmune disease, although it has still to be proved that the immune response was initiated in response to these same antigens.

Some autoimmune disorders may be triggered by infectious agents that express epitopes resembling self antigens and that lead to sensitization of the patient against that tissue. There is, however, also evidence from animal models of autoimmunity that many autoimmune disorders are caused by internal dysregulation of the immune system without the apparent participation of infectious agents.

15-9 Autoimmune diseases can be classified into clusters that are typically either organ-specific or systemic.

The classification of disease is an uncertain science, especially in the absence of a precise understanding of causative mechanisms. This is well illustrated by the difficulty in classifying the autoimmune diseases. From a clinical perspective it is often useful to distinguish between the following two major patterns of autoimmune disease: the diseases in which the expression of autoimmunity is restricted to specific organs of the body, known as 'organ-specific' autoimmune diseases; and those in which many tissues of the body are affected, the 'systemic' autoimmune diseases. In both types of autoimmunity, disease has a tendency to become chronic because, with a few notable exceptions (for example type 1 diabetes or Hashimoto's thyroiditis), the autoantigens are never cleared from the body. Some autoimmune diseases seem to be dominated by the pathogenic effects of a particular immune effector pathway, either autoantibodies or activated autoreactive T cells. However, both of these pathways often contribute to the overall pathogenesis of autoimmune disease.

In organ-specific diseases, autoantigens from one or a few organs only are targeted, and disease is therefore limited to those organs. Examples of organ-specific autoimmune diseases are **Hashimoto's thyroiditis** and **Graves' disease**, both predominantly affecting the thyroid gland, and type 1 diabetes, which is caused by immune attack on insulin-producing pancreatic β cells. Examples of systemic autoimmune disease are SLE and primary Sjögren's syndrome, in which tissues as diverse as the skin, kidneys, and brain may all be affected (Fig. 15.11).

The autoantigens recognized in these two categories of disease are themselves organ-specific and systemic, respectively. Thus, Graves' disease is characterized by the production of antibodies against the thyroid-stimulating hormone (TSH) receptor, which is specific to the thyroid gland, Hashimoto's thyroiditis by antibodies against thyroid peroxidase, and type 1 diabetes by anti-insulin antibodies. By contrast, SLE is characterized by the presence of antibodies against antigens that are ubiquitous and abundant in every cell of the body, such as chromatin and the proteins of the pre-mRNA splicing machinery—the spliceosome complex.

An unusual, but prevalent, variant of chronic inflammatory disease is **inflammatory bowel disease** (**IBD**), which includes two distinct clinical entities—Crohn's disease (discussed later in this chapter) and ulcerative colitis (see Section 15-7). We discuss IBD in this chapter because it has many features of an autoimmune disease, even though it is not primarily targeted against self-tissue antigens. Instead, the targets of the dysregulated immune response in IBD are antigens derived from the commensal microbiota resident in the intestines. Strictly speaking, therefore, IBD is an outlier among autoimmune diseases in that the immune response is not directed against 'self' antigens; rather, it is directed against microbial antigens of the resident, or 'self,'

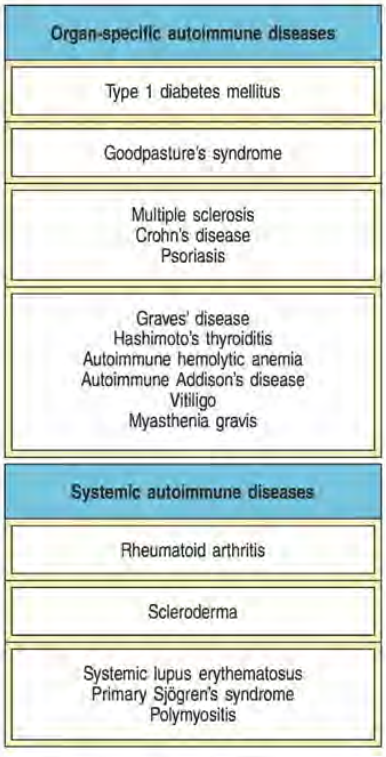


Fig. 15.11 Some common autoimmune diseases classified according to their 'organ-specific' or 'systemic' nature. Diseases that tend to occur in clusters are grouped in single boxes. Clustering is defined as more than one disease affecting a single patient or different members of a family. Not all autoimmune diseases can be classified according to this scheme. For example, autoimmune hemolytic anemia can occur in isolation or in association with systemic lupus erythematosus.

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microbiota. Nevertheless, features of immune tolerance breakdown characteristic of other autoimmune diseases are seen in IBD, and, in common with the organ-specific autoimmune diseases, the tissue destruction wrought by the aberrant immune response is primarily, although not exclusively, localized to a single organ—the intestines.

It is likely that the organ-specific and systemic autoimmune diseases have somewhat different etiologies, which provides a biological basis for their division into two broad categories. Evidence for the validity of this classification also comes from observations that different autoimmune diseases cluster within individuals and within families. The organ-specific autoimmune diseases frequently occur together in many combinations; for example, autoimmune thyroid disease and the autoimmune depigmenting disease vitiligo are often found in the same person. Similarly, SLE and Sjögren's syndrome can coexist within a single individual or among different members of a family.

These clusters of autoimmune diseases provide the most useful classification into different subtypes, each of which may turn out to have a distinct mechanism. The classification of autoimmune diseases given in Fig. 15.11 is based on such clustering. A strict separation of diseases into organ-specific and systemic categories does, however, break down to some extent, because not all autoimmune diseases can be usefully classified in this manner. For example, autoimmune hemolytic anemia, in which red blood cells are destroyed, sometimes occurs as a solitary entity and could be classified as an organ-specific disease. In other circumstances it can occur in conjunction with SLE as part of a systemic autoimmune disease.

15-10 Multiple components of the immune system are typically recruited in autoimmune disease.

Immunologists have long been concerned with the issue of which parts of the immune system are important in different autoimmune syndromes, because this can be useful in understanding how a disease is caused and how it is maintained, with the ultimate goal of finding effective therapies. In **myasthenia gravis**, for example, autoantibodies seem to have the main role in causing the disease symptoms. Antibodies produced against the acetylcholine receptor cause blocking of receptor function at the neuromuscular junction, resulting in a syndrome of muscle weakness. In other autoimmune conditions, antibodies in the form of immune complexes are deposited in tissues and cause tissue damage as a result of complement activation and ligation of Fc receptors on inflammatory cells, resulting in inflammation of the affected tissue.

Myasthenia Gravis



Relatively common autoimmune diseases in which effector T cells seem to be the main destructive agents include type 1 diabetes, psoriasis, IBD, and multiple sclerosis. In these diseases, T cells recognize self peptides or peptides derived from antigens of the commensal microbiota complexed with self-MHC molecules. The damage in such diseases is caused by the T cells recruiting and activating myeloid cells of the innate immune system (for example macrophages and neutrophils) to cause a local inflammation, or by direct T-cell damage to tissue cells. Affected tissues are heavily infiltrated by T lymphocytes and activated myeloid cells.

When disease can be transferred from a diseased individual to a healthy one by transferring autoantibodies and/or self-reactive T cells, this both confirms that the disease is autoimmune in nature and also proves the involvement of the transferred material in the pathological process. In myasthenia gravis, serum from affected patients can transfer similar disease symptoms to animal recipients, thus proving the pathogenic role of the anti-acetylcholine autoantibodies (Fig. 15.12). Similarly, in the animal model disease EAE, T cells from affected animals can transfer disease to normal animals (Fig. 15.13).

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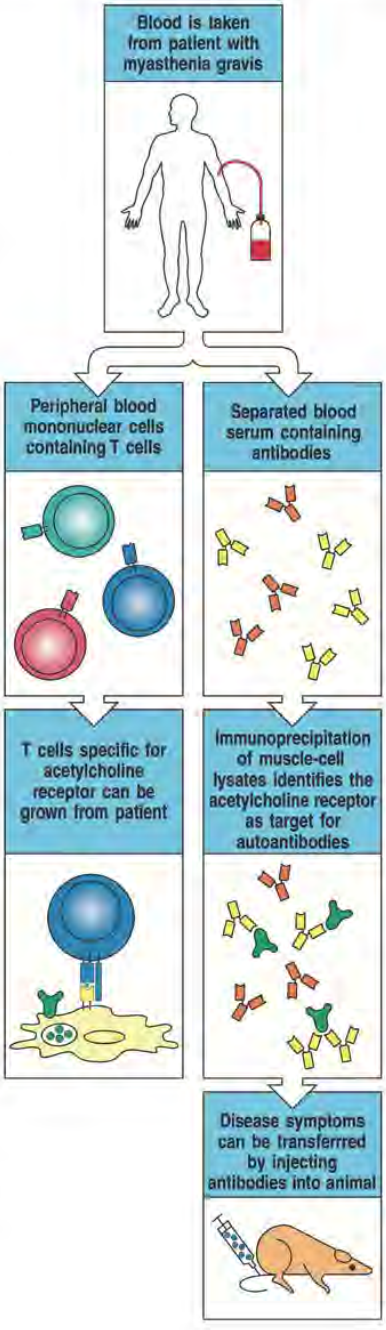


Fig. 15.12 Identification of autoantibodies that can transfer disease in patients with myasthenia gravis. Autoantibodies from the serum of patients with myasthenia gravis immunoprecipitate the acetylcholine receptor from lysates of skeletal muscle cells (right-hand panels). Because they can bind to both the murine and the human acetylcholine receptor, they can transfer disease when injected into mice (bottom panel). This experiment demonstrates that the antibodies are pathogenic. However, to be able to produce antibodies, the same patients should also have CD4 T cells that respond to a peptide derived from the acetylcholine receptor. To detect them, T cells from patients with myasthenia gravis are isolated and grown in the presence of the acetylcholine receptor plus antigen-presenting cells of the correct MHC type (left-hand panels). T cells specific for epitopes of the acetylcholine receptor are stimulated to proliferate and can thus be detected.

Pregnancy is an experiment of nature that can demonstrate a role for antibodies in the causation of disease. IgG antibodies, but not T cells, can cross the placenta (see Section 10-15). For some autoimmune diseases (Fig. 15.14), transmission of autoantibodies across the placenta leads to disease in the fetus or the neonate (Fig. 15.15). This provides proof in humans that such autoantibodies cause some of the symptoms of autoimmunity. The symptoms of disease in the newborn infant typically disappear rapidly as the maternal antibody is catabolized, but in some cases the antibodies cause chronic organ injury before they are removed, such as damage to the conducting tissue of the heart in babies of mothers with SLE or Sjögren's syndrome. Antibody clearance can be speeded up by exchange of the infant's blood or plasma (plasmapheresis), although this is of no clinical use after permanent injury has occurred, as in congenital heart block.

Although the diseases noted above are clear examples that a particular effector function, once established, can cause disease, the idea that most autoimmune diseases are caused solely by a single effector pathway of the immune system is an oversimplification. It is more useful to consider autoimmune responses, like immune responses to pathogens, as engaging the integrated immune system and therefore typically involving T cells, B cells, and innate immune cells. In the non-obese diabetic (NOD) mouse model of type 1 diabetes, for example—a disease that is usually considered to be T-cell mediated—B cells are required for disease initiation. In this case, the B cells are probably functioning as essential antigen-presenting cells for T cells, although the exact details are not clear. A selection of autoimmune diseases showing which parts of the immune response contribute to pathogenesis is given in Fig. 15.16.

15-11 Chronic autoimmune disease develops through positive feedback from inflammation, inability to clear the self antigen, and a broadening of the autoimmune response.

When normal immune responses are engaged to destroy a pathogen, the typical outcome is the elimination of the foreign invader, after which the immune response ceases, leaving only an expanded cohort of memory lymphocytes (see Chapter 11). In autoimmunity, however, the self antigen cannot easily be eliminated, because it is in vast excess or is ubiquitous, as with the SLE autoantigen, chromatin. Thus, a very important mechanism for limiting the extent of an immune response cannot apply to many autoimmune diseases. Instead, autoimmune diseases tend to evolve into a chronic state.

In general, autoimmune diseases are characterized by an early activation phase with the involvement of only a few autoantigens, followed by a chronic stage. The constant presence of autoantigen leads to chronic inflammation. This in turn leads to the release of more autoantigens as a result of tissue damage, and this breaks an important barrier to autoimmunity known

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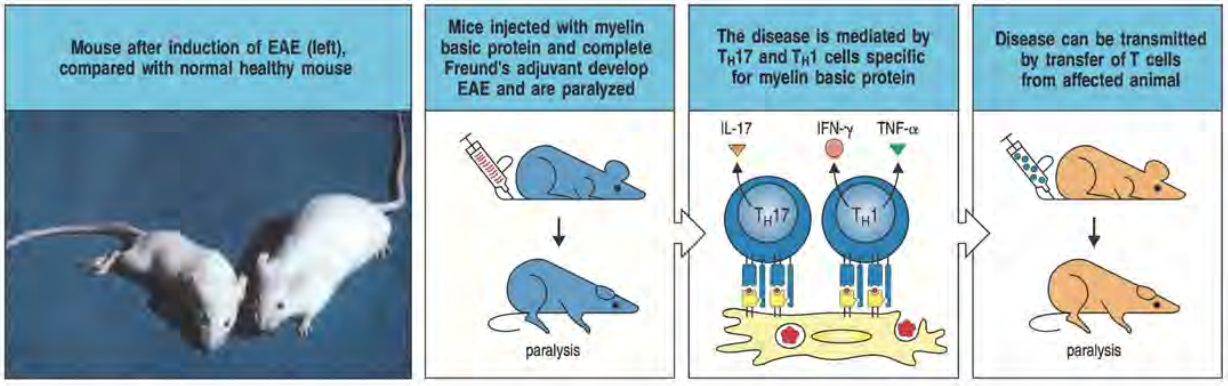


Fig. 15.13 T cells specific for myelin basic protein mediate inflammation of the brain in experimental autoimmune encephalomyelitis (EAE). This disease is produced in experimental animals by injecting them with isolated spinal cord homogenized in complete Freund's adjuvant. EAE is due to an inflammatory reaction in the brain that causes a progressive paralysis affecting first the tail and hind limbs (as shown in the mouse on the left of the photograph, compared with a healthy mouse on the right) before progressing to forelimb paralysis and eventual death. One of the autoantigens identified in the spinal cord homogenate is myelin basic protein (MBP). Immunization with MBP alone in complete Freund's adjuvant can also cause these disease symptoms. Inflammation of the brain and paralysis are mediated by T_H1 and T_H17 cells specific for MBP. Cloned MBP-specific T_H1 cells can transfer symptoms of EAE to naive recipients provided that the recipients carry the correct MHC allele. In this system it has therefore proved possible to identify the peptide:MHC complex recognized by the T_H1 clones that transfer disease. Other purified components of the myelin sheath can also induce the symptoms of EAE, so there is more than one autoantigen in this disease. Photograph from Wraith, D. *et al.*: *Cell* 1989, 59:247–255. With permission from Elsevier.

as 'sequestration,' by which many self antigens are normally kept apart from the immune system. It also leads to the attraction of nonspecific effector cells such as macrophages and neutrophils that respond to the release of cytokines and chemokines from injured tissues (Fig. 15.17). The result is a continuing and evolving self-destructive process.

The transition to the chronic stage is usually accompanied by an extension of the autoimmune response to new epitopes on the initiating autoantigen, and to new autoantigens. This phenomenon is known as **epitope spreading** and is important in perpetuating and amplifying the disease. As we saw in Chapter 10, activated B lymphocytes can efficiently internalize their cognate antigens by receptor-mediated endocytosis via their antigen receptor, process them and present the derived peptides to T cells. Epitope spreading can

Fig. 15.14 Some autoimmune diseases that can be transferred across the placenta by pathogenic IgG autoantibodies. These diseases are caused mostly by autoantibodies against cell-surface or tissue-matrix molecules. This suggests that an important factor determining whether an autoantibody that crosses the placenta causes disease in the fetus or newborn baby is the accessibility of the antigen to the autoantibody. Autoimmune congenital heart block is caused by fibrosis of the developing cardiac conducting tissue, which expresses abundant Ro antigen. Ro protein is a constituent of an intracellular small cytoplasmic ribonucleoprotein. It is not yet known whether it is expressed at the cell surface of cardiac conducting tissue to act as a target for autoimmune tissue injury. Nevertheless, autoantibody binding leads to tissue damage and results in slowing of the heart rate (bradycardia).

Autoimmune diseases transferred across the placenta to the fetus and newborn infant		
Disease	**Autoantibody**	**Symptom**
Myasthenia gravis	Anti-acetylcholine receptor	Muscle weakness
Graves' disease	Anti-thyroid-stimulating-hormone (TSH) receptor	Hyperthyroidism
Thrombocytopenic purpura	Anti-platelet antibodies	Bruising and hemorrhage
Neonatal lupus rash and/or congenital heart block	Anti-Ro antibodies Anti-La antibodies	Photosensitive rash and/or bradycardia
Pemphigus vulgaris	Anti-desmoglein-3	Blistering rash

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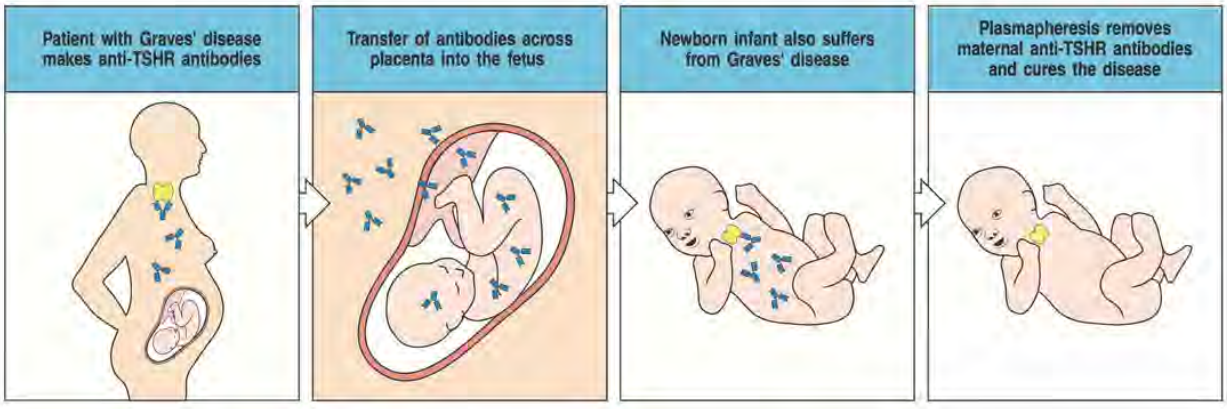


Fig. 15.15 Antibody-mediated autoimmune diseases can appear in the infants of affected mothers as a consequence of transplacental antibody transfer. In pregnant women, IgG antibodies cross the placenta and accumulate in the fetus before birth (see Fig. 10.24). Babies born to mothers with IgG-mediated autoimmune disease therefore frequently show symptoms similar to those of the mother in the first few weeks of life. Fortunately, there is little lasting damage because the symptoms disappear along with the maternal antibody. In Graves' disease, the symptoms are caused by antibodies against the thyroid-stimulating hormone receptor (TSHR). Children of mothers making thyroid-stimulating antibody are born with hyperthyroidism, but this can be corrected by replacing the plasma with normal plasma (plasmapheresis), thus removing the maternal antibody.

occur in several ways. Processing of the internalized autoantigen will reveal a variety of novel, previously hidden, peptide epitopes called **cryptic epitopes**, that the B cell can then present to T cells. Autoreactive T cells responding to these 'new' epitopes will provide help to any B cells presenting these peptides, recruiting additional B-cell clones to the autoimmune reaction, and resulting in the production of a greater variety of autoantibodies. In addition, on binding and internalizing specific antigen via their B-cell receptor, B cells will also internalize any other molecules closely associated with that antigen. By these routes, B cells can act as antigen-presenting cells for peptides derived from proteins completely different from the original autoantigen that initiated the autoimmune reaction.

The autoantibody response in SLE initiates these mechanisms of epitope spreading. In this disease, autoantibodies against both the protein and DNA components of chromatin are found. Figure 15.18 shows how autoreactive B cells specific for DNA can recruit autoreactive T cells specific for histone proteins, another component of chromatin, into the autoimmune response. In

Autoimmune diseases involve all aspects of the immune response			
Disease	**T cells**	**B cells**	**Antibody**
Systemic lupus erythematosus	Pathogenic Help for antibody	Present antigen to T cells	Pathogenic
Type 1 diabetes	Pathogenic	Present antigen to T cells	Present, but role unclear
Myasthenia gravis	Help for antibody	Antibody secretion	Pathogenic
Multiple sclerosis	Pathogenic	Present antigen to T cells	Present, but role unclear

Fig. 15.16 Autoimmune diseases involve all aspects of the immune response. Although some autoimmune diseases have traditionally been thought to be mediated by B cells or T cells, it is useful to consider that, typically, all aspects of the immune system have a role. For four important autoimmune diseases, the figure lists the roles of T cells, B cells, and antibody. In some diseases, such as SLE, T cells can have multiple roles such as helping B cells to make autoantibody and directly promoting tissue damage, whereas B cells can have two roles as well—presenting autoantigens to stimulate T cells and secreting pathogenic autoantibodies.

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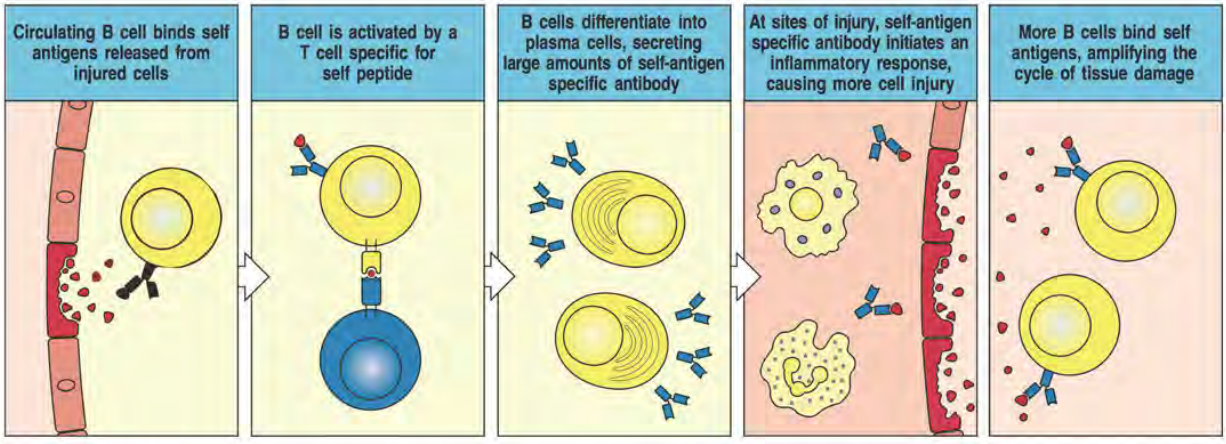


Fig. 15.17 Autoantibody-mediated inflammation can lead to the release of autoantigens from damaged tissues, which in turn promotes further activation of autoreactive B cells. Autoantigens, particularly intracellular ones that are targets in SLE, stimulate B cells only when released from dying cells (first panel). The result is the activation of autoreactive T and B cells and the eventual secretion of autoantibodies (second and third panels). These autoantibodies can mediate tissue damage through a variety of effector functions (see Chapter 10) and this results in the further death of cells (fourth panel). A positive feedback loop is established because these additional autoantigens recruit and activate additional autoreactive B cells (fifth panel). These in turn can start the cycle over again, as shown in the first panel.

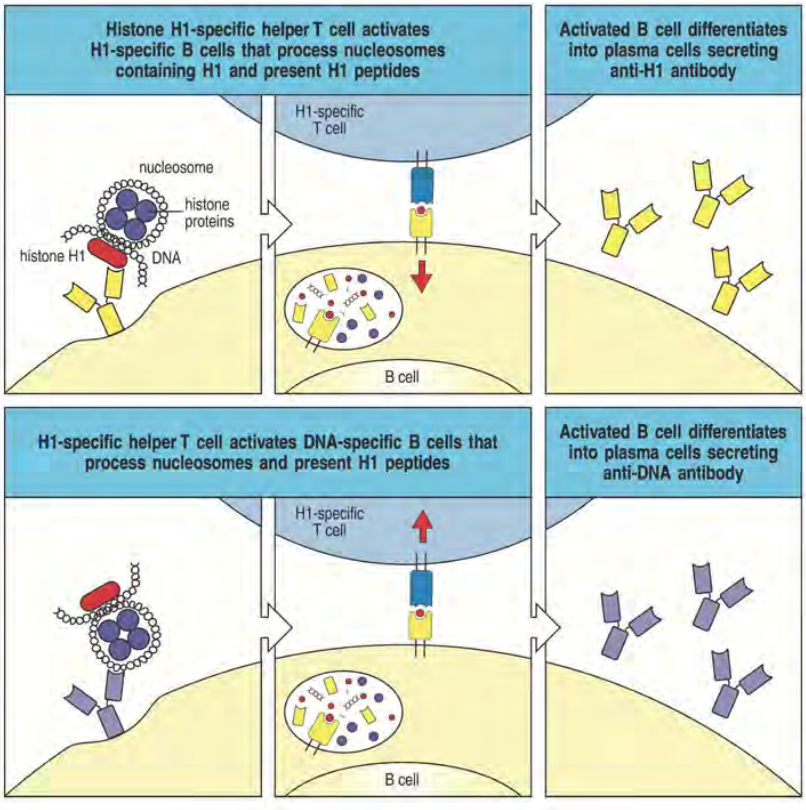


Fig. 15.18 Epitope spreading occurs when B cells specific for various components of a complex antigen are stimulated by an autoreactive helper T cell of a single specificity. In SLE, patients often produce autoantibodies against both the DNA and histone protein components of a nucleosome (a subunit of chromatin), or of some other complex antigen. The most likely explanation is that different autoreactive B cells have been activated by a single clone of autoreactive T cells specific for a peptide of one of the proteins in the complex. A B cell binding to any component of the complex through its surface immunoglobulin can internalize the whole complex, degrade it, and return peptides derived from the histone proteins to the cell surface bound to MHC class II molecules, where they stimulate helper T cells. These, in turn, activate the B cells. Thus, a T cell specific for the H1 histone protein of the nucleosome can activate both a B cell specific for H1 (upper panels) and a B cell specific for double-stranded DNA (lower panels). T cells of additional epitope specificities can also become recruited into the response in this way by antigen-presenting B cells bearing a variety of nucleosome-derived peptide:MHC complexes on their surface.

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turn, these T cells provide help not only to the original DNA-specific B cells but also to histone-specific B cells, resulting in the production of both anti-DNA and anti-histone antibodies.


Pemphigus Vulgaris

An autoimmune disease in which epitope spreading is linked to the progression of disease is **pemphigus vulgaris**, which is characterized by severe blistering of the skin and mucosal membranes. It is caused by autoantibodies against desmogleins, a type of cadherin present in cell junctions (desmosomes) that hold the cells of the epidermis together. Binding of autoantibodies to the extracellular domains of these adhesion molecules causes dissociation of the junctions and dissolution of the affected tissue. Pemphigus vulgaris usually starts with lesions in the oral and genital mucosa; only later does the skin become involved. In the mucosal stage, only autoantibodies against certain epitopes on desmoglein Dsg-3 are found, and these antibodies seem unable to cause skin blistering. Progression to the skin disease is associated both with epitope spreading within Dsg-3, which gives rise to autoantibodies that can cause deep skin blistering, and to another desmoglein, Dsg-1, which is more abundant in the epidermis. Dsg-1 is also the autoantigen in a less severe variant of the disease, pemphigus foliaceus. In that disease, the autoantibodies first produced against Dsg-1 cause no damage; disease appears only after autoantibodies are made against epitopes on parts of the protein involved in the adhesion of epidermal cells.

15-12 Both antibody and effector T cells can cause tissue damage in autoimmune disease.

The manifestations of autoimmune disease are caused by the effector mechanisms of the immune system being directed at the body's own tissues. As discussed previously, the response is usually amplified and maintained by the constant supply of new autoantigen. An important exception to this general rule is type 1 diabetes, in which the autoimmune response destroys the target organ completely. This leads to a failure to produce insulin—one of the major autoantigens in this disease—and it is the lack of insulin that is responsible for the disease symptoms.

The mechanisms of tissue injury in autoimmunity can be classified according to the scheme adopted for hypersensitivity reactions (Fig. 15.19; see also Fig. 14.1). It should be emphasized, however, that both B and T cells are involved in most autoimmune diseases, even in cases where a particular type of response predominates in causing tissue damage. The antigen, or group of antigens, against which the autoimmune response is directed, and the mechanism by which the antigen-bearing tissue is damaged, together determine the pathology and clinical expression of the disease.

Type I IgE-mediated hypersensitive responses play no major part in autoimmunity. By contrast, autoimmunity that damages tissues by mechanisms analogous to type II hypersensitivity reactions is quite common. In this form of autoimmunity, IgG or IgM responses to autoantigens located on cell surfaces or extracellular matrix cause the injury. In other autoimmune diseases, tissue damage is due primarily to type III responses involving the deposition of immune complexes (see Fig. 15.19); in autoimmunity, the immune complexes are composed of soluble autoantigens and their cognate autoantibodies. These autoimmune diseases are systemic and are characterized by autoimmune vasculitis—inflammation of blood vessels. In SLE, autoantibodies cause damage by both type II and type III mechanisms. Finally, several organ-specific autoimmune diseases are due to a type IV response in which T_H1 cells and/or cytotoxic T cells are directly involved in causing tissue damage.

In most autoimmune diseases, however, several mechanisms of immunopathogenesis operate. Notably, helper T cells are almost always required

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for the production of pathogenic autoantibodies. Reciprocally, B cells often have an important role in the maximal activation of T cells that mediate tissue damage or help autoantibody production (see Section 15-10). In type 1 diabetes and rheumatoid arthritis, for example, which are classed as T cell-mediated diseases, both T-cell and antibody-mediated pathways cause tissue injury. SLE is an example of an autoimmune disease that was previously thought to be mediated solely by antibodies and immune complexes but is now known to have a component of T cell-mediated pathogenesis as well. We will first examine how autoantibodies cause tissue damage, before considering self-reactive T-cell responses and their role in autoimmune disease.

Fig. 15.19 Mechanisms of tissue damage in autoimmune diseases. Autoimmune diseases can be grouped in the same way as hypersensitivity reactions, according to the predominant type of immune response and the mechanism by which it damages tissues. The immunopathological mechanisms given here are those illustrated for the hypersensitivity reactions in Fig. 14.1; type I IgE-mediated responses are not given here because they are not a known cause of autoimmune disease. Some additional autoimmune diseases in which the antigen is a cell-surface receptor, and in which the pathology is due to altered signaling, are listed later, in Fig. 15.23. In many autoimmune diseases, several immunopathogenic mechanisms operate in parallel. This is illustrated here for rheumatoid arthritis, which appears in more than one category of immunopathogenic mechanism.

Some common autoimmune diseases classified by immunopathogenic mechanism

Syndrome	Autoantigen	Consequence
Type II antibody against cell-surface or matrix antigens		
Autoimmune hemolytic anemia	Rh blood group antigens, I antigen	Destruction of red blood cells by complement and FcR^+ phagocytes, anemia
Autoimmune thrombocytopenic purpura	Platelet integrin GpIIb:IIIa	Abnormal bleeding
Goodpasture's syndrome	Noncollagenous domain of basement membrane collagen type IV	Glomerulonephritis, pulmonary hemorrhage
Pemphigus vulgaris	Epidermal cadherin	Blistering of skin
Acute rheumatic fever	Streptococcal cell-wall antigens. Antibodies cross-react with cardiac muscle	Arthritis, myocarditis, late scarring of heart valves
Type III immune-complex disease		
Mixed essential cryoglobulinemia	Rheumatoid factor IgG complexes (with or without hepatitis C antigens)	Systemic vasculitis
Rheumatoid arthritis	Rheumatoid factor IgG complexes	Arthritis
Type IV T-cell-mediated disease		
Type 1 diabetes	Pancreatic β-cell antigen	β-cell destruction
Rheumatoid arthritis	Unknown synovial joint antigen	Joint inflammation and destruction
Multiple sclerosis	Myelin basic protein, proteolipid protein, myelin oligodendrocyte glycoprotein	Brain invasion by CD4 T cells, muscle weakness, and other neurological symptoms
Crohn's disease	Antigens of intestinal microbiota	Regional intestinal inflammation and scarring
Psoriasis	Unknown skin antigens	Inflammation of skin with formation of plaques

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15-13 Autoantibodies against blood cells promote their destruction.

Autoimmune Hemolytic Anemia

IgG or IgM responses to antigens located on the surface of blood cells lead to the rapid destruction of these cells. An example of this is **autoimmune hemolytic anemia**, in which antibodies against self antigens on red blood cells trigger destruction of the cells, leading to anemia. This can occur in two ways (Fig. 15.20). Red cells with bound IgG or IgM antibody are rapidly cleared from the circulation by interaction with Fc or complement receptors, respectively, on cells of the fixed mononuclear phagocytic system; this occurs particularly in the spleen. Alternatively, the autoantibody-sensitized red cells are lysed by formation of the membrane-attack complex of complement. In **autoimmune thrombocytopenic purpura**, autoantibodies against the GpIIb:IIIa fibrinogen receptor or other platelet-specific surface antigens can cause thrombocytopenia (a depletion of platelets), which can in turn cause hemorrhage.

Lysis of nucleated cells by complement is less common because these cells are better defended by complement regulatory proteins, which protect cells against immune attack by interfering with the activation of complement components and their assembly into a membrane-attack complex (see Section 2-15). Nevertheless, nucleated cells targeted by autoantibodies are still destroyed by cells of the mononuclear phagocytic system. Autoantibodies against neutrophils, for example, cause neutropenia, which increases susceptibility to infection with pyogenic bacteria. In all these cases, accelerated clearance of autoantibody-sensitized cells is the cause of their depletion in the blood. One therapeutic approach to this type of autoimmunity is removal of the spleen, the organ in which the main clearance of red cells, platelets, and leukocytes occurs. Another is the administration of large quantities of nonspecific IgG (termed IVIG, for intravenous immunoglobulin), which among other mechanisms inhibits the Fc receptor-mediated uptake of antibody-coated cells.

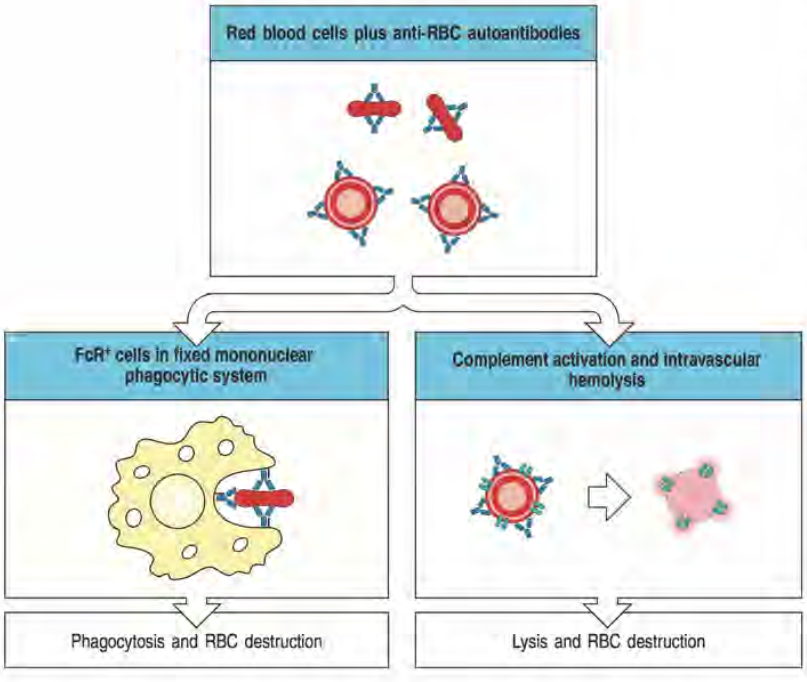


Fig. 15.20 Antibodies specific for cell-surface antigens can destroy cells. In autoimmune hemolytic anemias, red blood cells (RBCs) coated with IgG autoantibodies against a cell-surface antigen are rapidly cleared from the circulation by uptake by Fc receptor-bearing macrophages in the fixed mononuclear phagocytic system (left panel). Red cells coated with IgM autoantibodies fix C3 and are cleared by CR1- and CR3-bearing macrophages in the fixed mononuclear phagocytic system (not shown). Uptake and clearance by these mechanisms occurs mainly in the spleen. The binding of certain rare autoantibodies that fix complement extremely efficiently causes the formation of the membrane-attack complex on the red cells, leading to intravascular hemolysis (right panel).

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15-14 The fixation of sublytic doses of complement to cells in tissues stimulates a powerful inflammatory response.

The binding of IgG and IgM antibodies to cells in tissues causes inflammatory injury by a variety of mechanisms. One of these is the fixation of complement. Although nucleated cells are relatively resistant to lysis by complement, the assembly of sublytic amounts of the membrane-attack complex on their surface provides a powerful activating stimulus. Depending on the type of cell, this interaction can cause cytokine release, the generation of a respiratory burst, or the mobilization of membrane phospholipids to generate arachidonic acid—the precursor of prostaglandins and leukotrienes, which are lipid mediators of inflammation.

Most cells in tissues are fixed in place, and innate and adaptive immune cells are attracted to them by chemoattractant molecules. One such molecule is the complement fragment C5a, which is released as a result of complement activation triggered by autoantibody binding. Other chemoattractants, such as leukotriene B4, can be released by the autoantibody-targeted cells. Inflammatory leukocytes are further activated by binding to autoantibody Fc regions and fixed complement C3 fragments on the tissue cells. Tissue injury can then result from the products of the activated leukocytes and by antibody-dependent cellular cytotoxicity mediated by NK cells (see Section 10-23).

A probable example of this type of autoimmunity is Hashimoto's thyroiditis, in which autoantibodies against tissue-specific antigens such as thyroid peroxidase and thyroglobulin are found at extremely high levels for prolonged periods. Direct T cell-mediated cytotoxicity, which we discuss later, is probably also important in this disease.

15-15 Autoantibodies against receptors cause disease by stimulating or blocking receptor function.

A special class of type II hypersensitivity reaction occurs when the autoantibody binds to a cell-surface receptor. Antibody binding to a receptor can either stimulate the receptor or block its stimulation by its natural ligand. In Graves' disease, autoantibody against the thyroid-stimulating hormone receptor on thyroid cells stimulates the excessive production of thyroid hormone. The production of thyroid hormone is normally controlled by feedback regulation; high levels of thyroid hormone inhibit the release of thyroid-stimulating hormone (TSH) by the pituitary. In Graves' disease, feedback inhibition fails because the autoantibody continues to stimulate the TSH receptor in the absence of TSH, and the patient becomes hyperthyroid (Fig. 15.21).

Myasthenia Gravis

In myasthenia gravis, autoantibodies against the α chain of the nicotinic acetylcholine receptor, which is present on skeletal muscle cells at neuromuscular junctions, can block neuromuscular transmission. The antibodies are believed to drive the internalization and intracellular degradation of acetylcholine receptors (Fig. 15.22). Patients with myasthenia gravis develop potentially fatal progressive weakness as a result of their autoimmune disease. Diseases caused by autoantibodies that act as agonists or antagonists for cell-surface receptors are listed in Fig. 15.23.

15-16 Autoantibodies against extracellular antigens cause inflammatory injury by mechanisms akin to type II and type III hypersensitivity reactions.

Antibody responses to extracellular matrix molecules are infrequent, but they can be very damaging when they occur. In **Goodpasture's syndrome**, an example of a type II hypersensitivity reaction (see Fig. 14.1), antibodies are

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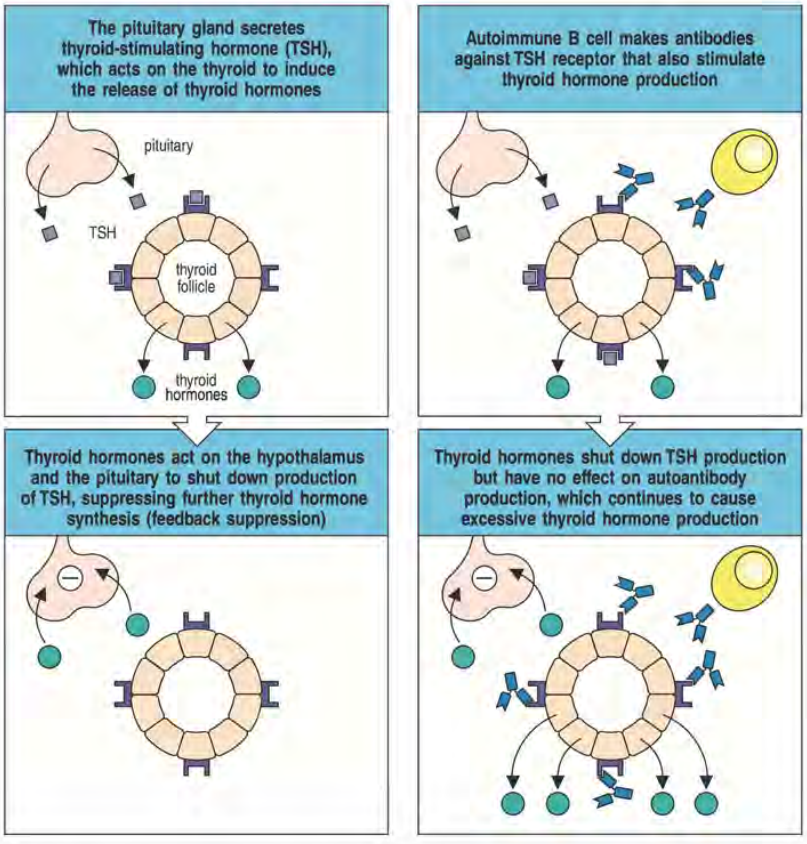


Fig. 15.21 Feedback regulation of thyroid hormone production is disrupted in Graves' disease. Graves' disease is caused by autoantibodies specific for the receptor for thyroid-stimulating hormone (TSH). Normally, thyroid hormones are produced in response to TSH and limit their own production by inhibiting the production of TSH by the pituitary (left panels). In Graves' disease, the autoantibodies are agonists for the TSH receptor and therefore stimulate the production of thyroid hormones (right panels). The thyroid hormones inhibit TSH production in the normal way but do not affect production of the autoantibody; the excessive thyroid hormone production induced in this way causes hyperthyroidism.

formed against the α_3 chain of basement membrane collagen (type IV collagen). These antibodies bind to the basement membranes of renal glomeruli (Fig. 15.24a) and, in some cases, to the basement membranes of pulmonary alveoli, causing a rapidly fatal disease if untreated. The autoantibodies bound to basement membrane ligate Fcγ receptors, leading to the activation of monocytes, neutrophils, and tissue basophils and mast cells. These release chemokines that attract a further influx of neutrophils into glomeruli, causing severe tissue injury (Fig. 15.24b). The autoantibodies also cause a local activation of complement, which may amplify the tissue injury.

Immune complexes are produced whenever there is an antibody response to a soluble antigen (see Appendix I, Section A-8). They are normally cleared efficiently by red blood cells bearing complement receptors and by phagocytes of the mononuclear phagocytic system that have both complement and

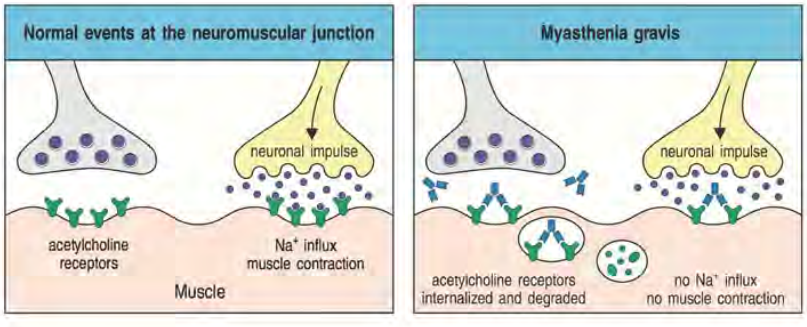


Fig. 15.22 Autoantibodies inhibit receptor function in myasthenia gravis. In normal circumstances, acetylcholine released from stimulated motor neurons at the neuromuscular junction binds to acetylcholine receptors on skeletal muscle cells, triggering muscle contraction (left panel). Myasthenia gravis is caused by autoantibodies against the α subunit of the receptor for acetylcholine. These autoantibodies bind to the receptor without activating it and also cause receptor internalization and degradation (right panel). As the number of receptors on the muscle is decreased, the muscle becomes less responsive to acetylcholine.

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Fig. 15.23 Autoimmune diseases caused by autoantibodies against cell-surface receptors. These antibodies produce different effects depending on whether they are agonists (which stimulate the receptor) or antagonists (which inhibit it). Note that different autoantibodies against the insulin receptor can either stimulate or inhibit signaling.

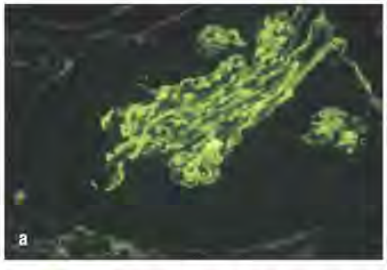
Diseases mediated by autoantibodies against cell-surface receptors		
Syndrome	**Antigen**	**Consequence**
Graves' disease	Thyroid-stimulating hormone receptor	Hyperthyroidism
Myasthenia gravis	Acetylcholine receptor	Progressive weakness
Insulin-resistant diabetes (type 2 diabetes)	Insulin receptor (antagonist)	Hyperglycemia, ketoacidosis
Hypoglycemia	Insulin receptor (agonist)	Hypoglycemia
Chronic urticaria	Receptor-bound IgE or IgE receptor (agonist)	Persistent itchy rash

Fc receptors, and such complexes cause little tissue damage. This clearance system can, however, fail in three circumstances. The first follows the injection of large amounts of antigen, leading to the formation of large amounts of immune complexes that overwhelm the normal clearance mechanisms. An example of this is serum sickness (see Section 14-16), which is caused by the injection of large amounts of serum proteins or by small-molecule drugs binding to serum proteins and acting as haptens. Serum sickness is a transient disease, lasting only until the immune complexes have been cleared. The second circumstance is seen in chronic infections such as bacterial endocarditis, in which the immune response to bacteria lodged on a cardiac valve is incapable of clearing the infection. The persistent release of bacterial antigens from the valve infection in the presence of a strong antibacterial antibody response causes widespread immune-complex injury to small blood vessels in organs such as the kidney and the skin. Chronic infections, such as hepatitis C infection, can lead to the production of cryoglobulins and the condition **mixed essential cryoglobulinemia**, in which immune complexes are deposited in joints and tissues.

Drug-Induced Serum Sickness

Mixed Essential Cryoglobulinemia

Third, part of the pathogenesis of SLE can also be attributed to the failure to clear immune complexes. In SLE there is chronic IgG antibody production directed at ubiquitous self antigens present in all nucleated cells, leading to a wide range of autoantibodies against common cellular constituents. The main antigens are three types of intracellular nucleoprotein particles—the nucleosome subunits of chromatin, the spliceosome, and a small cytoplasmic ribonucleoprotein complex containing two proteins known as Ro and La (named after the first two letters of the surnames of the two patients in whom autoantibodies against these proteins were discovered). For these autoantigens to participate in immune-complex formation, they must become extracellular.



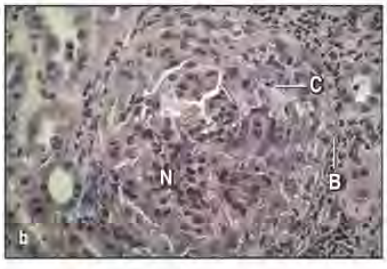


Fig. 15.24 Autoantibodies reacting with glomerular basement membrane cause the inflammatory glomerular disease known as Goodpasture's syndrome. The panels show sections of renal glomeruli in serial biopsies taken from patients with Goodpasture's syndrome. Panel a: glomerulus stained for IgG deposition by immunofluorescence. Anti-glomerular basement membrane antibody (stained green) is deposited in a linear fashion along the glomerular basement membrane. The autoantibody causes local activation of cells bearing Fc receptors, complement activation, and influx of neutrophils. Panel b: hematoxylin and eosin staining of a section through a renal glomerulus shows that the glomerulus is compressed by the formation of a crescent (C) of proliferating mononuclear cells within the Bowman's capsule (B) and there is an influx of neutrophils (N) into the glomerular tuft. Photographs courtesy of M. Thompson and D. Evans.

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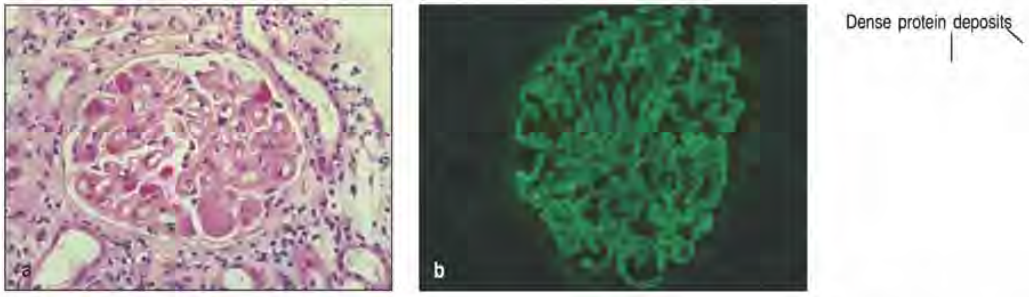


Fig. 15.25 Deposition of immune complexes in the renal glomerulus causes renal failure in systemic lupus erythematosus (SLE). Panel a: a section through a renal glomerulus from a patient with SLE, showing that the deposition of immune complexes has caused thickening of the glomerular basement membrane, seen as the clear 'canals' running through the glomerulus. Panel b: a similar section stained with fluorescent anti-immunoglobulin, revealing immunoglobulin deposits in the basement membrane. In panel c, the immune complexes are seen under the electron microscope as dense protein deposits between the glomerular basement membrane and the renal epithelial cells. Polymorphonuclear neutrophilic leukocytes are also present, attracted by the deposited immune complexes. Photographs courtesy of H.T. Cook and M. Kashgarian.



Systemic Lupus Erythematosus

The autoantigens of SLE are exposed on dead and dying cells and are released from injured tissues. In SLE, large quantities of antigen are available, so large amounts of small immune complexes are produced continuously and are deposited in the walls of small blood vessels in the renal glomerulus, in glomerular basement membrane (Fig. 15.25), in joints, and in other organs. This leads to the activation of phagocytic cells through their Fc receptors. The consequent tissue damage releases more nucleoprotein complexes, which in turn form more immune complexes. During this process, autoreactive T cells also become activated, although much less is known about their specificity. The experimental animal models for SLE cannot be initiated without the help of T cells, and T cells can also be directly pathogenic, forming part of the cellular infiltrates in skin and the interstitial areas of the kidney. As we discuss in the next section, T cells contribute to autoimmune disease in two ways: by helping B cells to make antibodies, in an analogous manner to a normal T-dependent immune response, and by direct effector functions of T cells as they infiltrate and destroy target tissues such as skin, renal interstitium, and vessels. Eventually, the inflammation induced in these tissues can cause sufficient damage to kill the patient.

15-17 T cells specific for self antigens can cause direct tissue injury and sustain autoantibody responses.

It is much more difficult to demonstrate the existence of autoreactive T cells than it is to demonstrate the presence of autoantibodies. First, autoreactive human T cells cannot be used to transfer disease to experimental animals because T-cell recognition is MHC-restricted, and animals and humans have different MHC alleles. Second, it is difficult to identify the antigen recognized by a T cell; for example, autoantibodies can be used to stain self tissues to reveal the distribution of the autoantigen, whereas T cells cannot be used in the same way. Nevertheless, there is strong evidence for the involvement of autoreactive T cells in several autoimmune diseases. In type 1 diabetes, for example, the insulin-producing β cells of the pancreatic islets of Langerhans are selectively destroyed by specific cytotoxic T cells. In rare cases in which patients with diabetes were transplanted with half a pancreas from an identical twin donor, the β cells in the grafted tissue were rapidly and selectively destroyed by the recipient's T cells. Recurrence of disease can be prevented by the immunosuppressive drug cyclosporin A (see Chapter 16), which inhibits T-cell activation.

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Autoantigens recognized by CD4 T cells can be identified by adding cells or tissues, containing autoantigens, to cultures of blood mononuclear cells and testing for recognition by CD4 cells derived from an autoimmune patient. If the autoantigen is present, it should be effectively presented, because phagocytes in the blood cultures can take up extracellular protein, degrade it in intracellular vesicles, and present the resulting peptides bound to MHC class II molecules. The identification of autoantigenic peptides is particularly difficult in autoimmune diseases in which CD8 T cells have a role, because autoantigens recognized by CD8 T cells are not effectively presented in such cultures. Peptides presented by MHC class I molecules must usually be made by the target cells themselves (see Chapter 6); intact cells of target tissue from the patient must therefore be used to study autoreactive CD8 T cells that cause tissue damage. Conversely, the pathogenesis of the disease can itself give clues to the identity of the antigen in some CD8 T cell-mediated diseases. For example, in type 1 diabetes, the insulin-producing β cells seem to be specifically targeted and destroyed by CD8 T cells (Fig. 15.26). This suggests that a protein unique to β cells is the source of the peptide recognized by the pathogenic CD8 T cells. Studies in the NOD mouse model of type 1 diabetes have shown that peptides from insulin itself are recognized by pathogenic CD8 cells, confirming insulin as one of the principal autoantigens in this diabetes model.

Multiple sclerosis is an example of a T cell-mediated chronic neurological disease that is caused by a destructive immune response against several brain antigens, including myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG). It takes its name from the hard (sclerotic) lesions, or plaques, that develop in the white matter of the central nervous system. These lesions show dissolution of the myelin that normally sheathes nerve cell axons, along with inflammatory infiltrates of lymphocytes and macrophages, particularly along the blood vessels. Patients with multiple sclerosis develop a variety of neurological symptoms, including muscle weakness, ataxia, blindness, and paralysis of the limbs. Lymphocytes and other blood cells do not normally cross the blood–brain barrier, but if the

Multiple Sclerosis

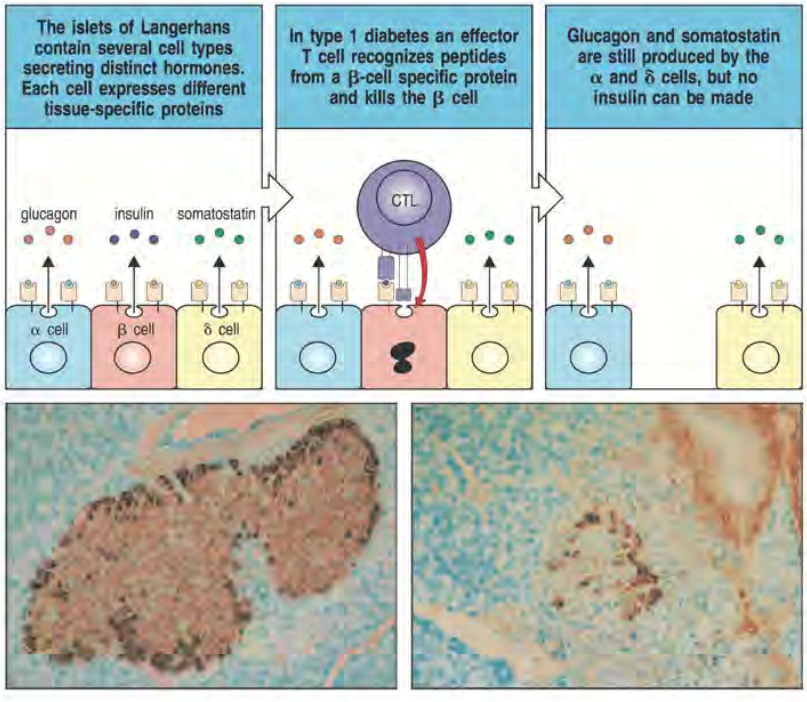


Fig. 15.26 Selective destruction of pancreatic β cells in type 1 diabetes indicates that the autoantigen is produced in β cells and recognized on their surface. In type 1 diabetes there is highly specific destruction of insulin-producing β cells in the pancreatic islets of Langerhans, sparing other islet cell types (α and δ). This is shown schematically in the upper panels. In the lower panels, islets from normal (left) and diabetic (right) mice are stained for insulin (brown), which shows the β cells, and for glucagon (black), which shows the α cells. Note the lymphocytes infiltrating the islet in the diabetic mouse (right) and the selective loss of the β cells (brown), whereas the α cells (black) are spared. The characteristic morphology of the islet is also disrupted with the loss of the β cells. Photographs courtesy of I. Visintin.

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brain and its blood vessels become inflamed, the blood–brain barrier breaks down. When this happens, activated CD4 T cells autoreactive for brain antigens and expressing α_4:β_1 integrin can bind vascular cell adhesion molecules (VCAM) on the surface of the activated venule endothelium (see Section 11-6), enabling the T cells to migrate out of the blood vessel. There they reencounter their specific autoantigen presented by MHC class II molecules on microglial cells (Fig. 15.27). Microglia are phagocytic macrophage-like cells of the innate immune system resident in the central nervous system and, like macrophages, can act as antigen-presenting cells. Inflammation causes increased vascular permeability and the site becomes heavily infiltrated by T_H17 and T_H1 cells, which produce IL-17 and IFN-γ, respectively. Cytokines and chemokines produced by the infiltrating effector T cells in turn recruit and activate myeloid cells that exacerbate the inflammation, resulting in the further recruitment of T cells, B cells, and innate immune cells to the site of the lesion. Autoreactive B cells produce autoantibodies against myelin antigens with help from T cells. These combined activities lead to demyelination and interference with neuronal function.

Rheumatoid arthritis (**RA**) is a chronic disease characterized by inflammation of the synovium (the thin lining of a joint). As the disease progresses, the inflamed synovium invades and damages the cartilage, followed by erosion of the bone (Fig. 15.28). Patients with rheumatoid arthritis suffer chronic pain, loss of function, and disability. Rheumatoid arthritis was at first considered an autoimmune disease driven mainly by B cells producing anti-IgG autoantibodies called rheumatoid factor (see Section 15-4). However, the identification of rheumatoid factor in some healthy individuals, and its absence in some patients with rheumatoid arthritis, suggested that more complex mechanisms orchestrate this pathology. The discovery that rheumatoid arthritis has an association with particular class II HLA-DR genes of the MHC suggested that T cells were involved in the pathogenesis of this disease. In rheumatoid arthritis, as in multiple sclerosis, autoreactive CD4 T cells become activated by dendritic cells and by inflammatory cytokines produced by macrophages. Once activated, the autoreactive T cells provide help to B cells to differentiate into plasma cells producing arthritogenic antibodies. Autoantigens such as type II collagen, proteoglycans, aggrecan, cartilage link protein, and heat-shock proteins have been proposed as potential antigens because of their ability to induce arthritis in mice. Their pathogenic role in humans remains to be ascertained, however. The activated T cells produce cytokines, which in turn stimulate monocytes/macrophages, endothelial cells, and fibroblasts to produce more pro-inflammatory cytokines such as TNF-α, IL-1 and IFN-γ, or chemokines (CXCL8, CCL2), and finally matrix metalloproteinases, which

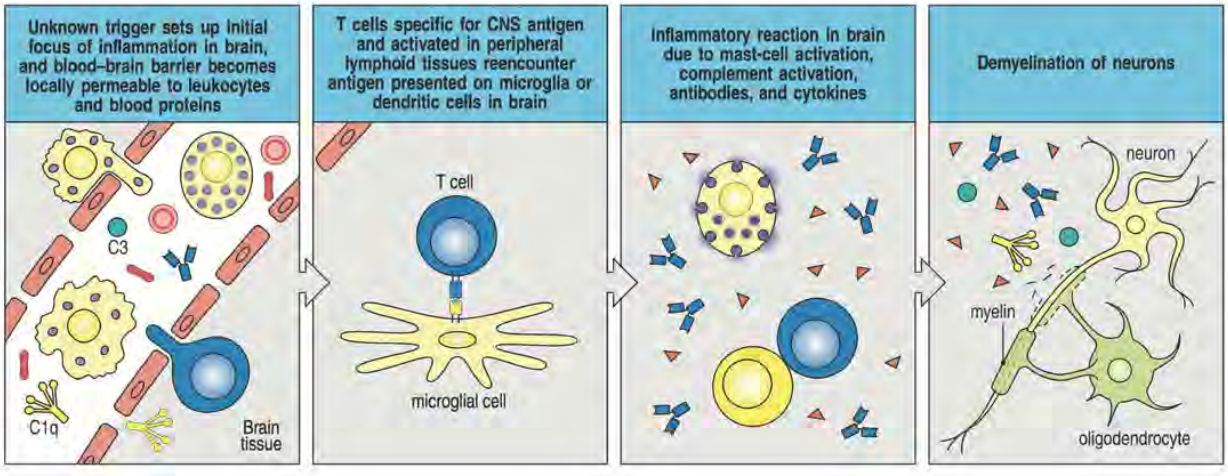


Fig. 15.27 The pathogenesis of multiple sclerosis. At sites of inflammation, activated T cells autoreactive for brain antigens can cross the blood–brain barrier and enter the brain, where they reencounter their antigens on microglial cells and secrete cytokines such as IFN-γ. The production of T-cell and macrophage cytokines exacerbates the inflammation and induces a further influx of blood cells (including macrophages, dendritic cells, and B cells) and blood proteins (such as complement) into the affected site. Mast cells also become activated. The individual roles of these components in demyelination and loss of neuronal function are still not well understood. CNS, central nervous system.

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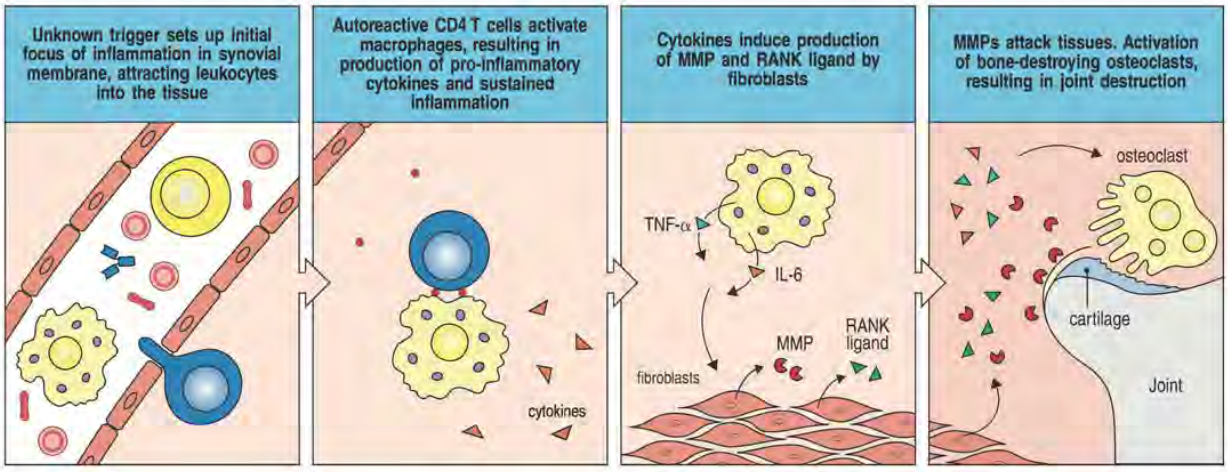


Fig. 15.28 The pathogenesis of rheumatoid arthritis. Inflammation of the synovial membrane, initiated by some unknown trigger, attracts autoreactive lymphocytes and macrophages to the inflamed tissue. Autoreactive effector CD4 T cells activate macrophages, with the production of pro-inflammatory cytokines such as IL-1, IL-6, IL-17, and TNF-α. Fibroblasts activated by cytokines produce matrix metalloproteinases (MMPs), which contribute to tissue destruction. The TNF family cytokine RANK ligand, expressed by T cells and fibroblasts in the inflamed joint, is the primary activator of bone-destroying osteoclasts. Antibodies against several joint proteins are also produced (not shown), but their role in pathogenesis is uncertain.

Rheumatoid Arthritis

Systemic Onset Juvenile Idiopathic Arthritis

are responsible for tissue destruction. Therapeutic antibodies against TNF-α have been successful in treating the symptoms of the disease (discussed in Section 16-8). However, it needs to be realized that in rheumatoid arthritis, as in many other autoimmune diseases, we do not yet know how disease starts. Mouse models of rheumatoid arthritis teach us that both T cells and B cells are needed to initiate the disease, because mice lacking T cells or B cells are resistant to its development.

Summary.

Autoimmune diseases can be broadly classified into those that affect a specific organ and those that affect tissues throughout the body. Organ-specific autoimmune diseases include diabetes, multiple sclerosis, psoriasis, Crohn's disease, myasthenia gravis, and Graves' disease. In each case the effector functions target autoantigens that are restricted to particular organs: insulin-producing β cells of the pancreas (diabetes), the myelin sheathing axons in the central nervous system (multiple sclerosis), and the thyroid-stimulating hormone receptor (Graves' disease), or, in the special case of Crohn's disease, components of the resident intestinal microbiota. In contrast, systemic diseases such as systemic lupus erythematosus (SLE) cause inflammation in multiple tissues because their autoantigens, which include chromatin and ribonucleoproteins, are found in every cell of the body. In some organ-specific diseases, immune destruction of the target tissue and the unique self antigens it expresses leads to cessation of autoimmune activity, but systemic diseases tend to be chronically active if untreated, because their autoantigens cannot be cleared. Another way of classifying autoimmune diseases is according to the effector functions that are most important in pathogenesis. It is becoming clear, however, that many diseases once thought to be mediated solely by one effector function actually involve several. In this way, autoimmune diseases resemble pathogen-directed immune responses, which typically elicit the activities of multiple effectors.

For a disease to be classified as autoimmune, the tissue damage must be shown to be caused by the adaptive immune response to self antigens.

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Autoinflammatory reactions directed against the commensal microbiota of the intestines, such as those that cause inflammatory bowel disease such as Crohn's, are a special case in that the target antigens are not strictly 'self,' but are derived from the 'self' intestinal microbiota. The IBDs do, nevertheless, share immunopathogenic features with other autoimmune diseases. The most convincing proof that the immune response is causal in autoimmunity is the transfer of disease by transferring the active component of the immune response to an appropriate recipient. Autoimmune diseases are mediated by autoreactive lymphocytes and/or their soluble products, pro-inflammatory cytokines, and autoantibodies responsible for inflammation and tissue injury. A few autoimmune diseases are caused by antibodies that bind to cell-surface receptors, causing either excess activity or inhibition of receptor function. In these diseases, transplacental passage of natural IgG autoantibodies can cause disease in the fetus and in the neonate. T cells can be involved directly in inflammation or cellular destruction, and they are also required to sustain an autoantibody response. Similarly, B cells are important antigen-presenting cells for sustaining autoantigen-specific T-cell responses and for causing epitope spreading. In spite of our knowledge of the mechanisms of tissue damage and the therapeutic approaches that this information has engendered, the deeper, more important question is how the autoimmune response is induced.

The genetic and environmental basis of autoimmunity.

Given the complex and varied mechanisms that exist to prevent autoimmunity, it is not surprising that autoimmune diseases are the result of multiple factors, both genetic and environmental. We first discuss the genetic basis of autoimmunity, attempting to understand how genetic defects perturb the various tolerance mechanisms. Genetic defects alone are not, however, always sufficient to cause autoimmune disease. Environmental factors such as toxins, drugs, and infections also play a part, although these factors are poorly understood. As we shall see, genetic and environmental factors together can overcome tolerance mechanisms and result in autoimmune disease.

15-18 Autoimmune diseases have a strong genetic component.

Although the causes of autoimmunity are still being worked out, it is increasingly clear that some individuals are genetically predisposed to autoimmunity. Perhaps the clearest demonstration of this is found in the several inbred mouse strains that are prone to various types of autoimmune diseases. For example, mice of the NOD strain are very likely to get diabetes. The female mice become diabetic more quickly than the males (Fig. 15.29). Many autoimmune diseases are more common in females than in males (see Fig. 15.33 below), although occasionally the opposite is true. Autoimmune diseases in humans also have a genetic component. Some autoimmune diseases, including type 1 diabetes, run in families, suggesting a role for genetic susceptibility. Most convincingly, if one of two identical (monozygotic) twins is affected, then the other twin is quite likely to be as well, whereas concordance of disease is much less in nonidentical (dizygotic) twins.

Environmental influences are also clearly involved. For example, although most of a colony of NOD mice are destined to get diabetes, they will do so at different ages (see Fig. 15.29). Moreover, the timing of disease onset often differs from one investigator's animal colony to the next, even though all the mice are genetically identical. Thus, environmental variables must be, in part, determining the rate of development of diabetes; a few even escape

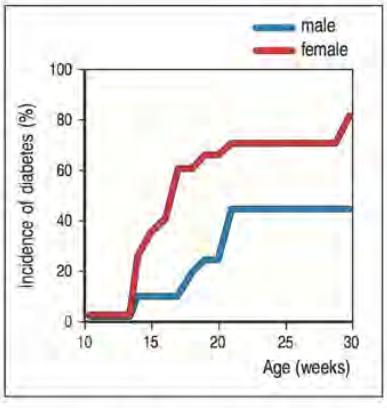


Fig. 15.29 Sex differences in the incidence of autoimmune disease. Many autoimmune diseases are more common in females than males, as illustrated here by the cumulative incidence of diabetes in a population of diabetes-prone NOD mice. Females (red line) get diabetes at a much younger age than do males, indicating their greater predisposition. Data kindly provided by S. Wong.

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the disease entirely. Particularly striking is the importance of the intestinal microbiota in the development of inflammatory bowel disease in genetically susceptible mice. Treatment with broad-spectrum antibiotics that reduce or eliminate many components of the commensal flora can delay or eliminate disease onset, and re-derivation of susceptible mice under germ-free conditions eliminates disease. Identical twins tell a similar story. With Crohn's disease, although disease incidence in susceptible monozygotic twins is much higher than in dizygotic twins, the concordance rate is far from 100%. The explanation for the incomplete concordance could lie in variability in the composition of the intestinal microbiota, or it could simply be random.

15-19 Several approaches have given us insight into the genetic basis of autoimmunity.

Since the advent of gene knockout technology in mice (see Section A-47, Appendix I), many genes that encode proteins of the immune system have been experimentally disrupted. Several of these mutant mouse strains have signs of autoimmune disease, including autoantibodies and, in some cases, infiltration of organs by T cells. The study of these mice has greatly expanded our knowledge of the genetic pathways that can contribute to autoimmunity and that therefore might be candidates for naturally occurring mutations. A growing number of genes whose deletion or overexpression can contribute to the pathogenesis of autoimmunity have been identified. These encode cytokines, co-receptors, members of cytokine- or antigen-signaling cascades, co-stimulatory molecules, proteins involved in pathways that promote apoptosis and those that inhibit it, and proteins that clear antigen or antigen:antibody complexes. Some of the cytokines and signaling proteins implicated in autoimmune disease are listed in Fig. 15.30, and Fig. 15.31 lists some of the known associations for other categories of proteins.

Fig. 15.30 Defects in cytokine production or signaling that can lead to autoimmunity. Some of the signaling pathways involved in autoimmunity have been identified by genetic analysis, mainly in animal models. The effects of overexpression or underexpression of some of the cytokines and intracellular signaling molecules involved are listed here (see the text for further discussion).

Defects in cytokine production or signaling that can lead to autoimmunity		
Defect	**Cytokine or intracellular signal**	**Result**
Overexpression	TNF-α	Inflammatory bowel disease, arthritis, vasculitis
	IL-2, IL-7, IL-2R	Inflammatory bowel disease
	IL-3	Demyelinating syndrome
	IFN-γ	Overexpression in skin leads to SLE
	STAT4	Inflammatory bowel disease
Underexpression	TNF-α	SLE
	IL-1 receptor agonist	Arthritis
	IL-10, IL-10R, STAT3	Inflammatory bowel disease
	TGF-β	Ubiquitous underexpression leads to inflammatory bowel disease. Underexpression specifically in T cells leads to SLE

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In humans, the association of autoimmunity with a particular gene or genetic region can be assessed by large-scale family studies, or by association studies in the general population (**genome-wide association studies**, or **GWASs**), that look for a correlation between disease frequency and variant alleles,

Proposed mechanism	Murine models	Disease phenotype	Human gene affected	Disease phenotype
Antigen clearance and presentation	C1q knockout	Lupus-like	*C1QA*	Lupus-like
	C4 knockout		*C2, C4*	
			Mannose-binding lectin	
	AIRE knockout	Multiorgan autoimmunity resembling APECED	*AIRE*	APECED
	Mer knockout	Lupus-like		
Signaling	SHP-1 knockout	Lupus-like		
	Lyn knockout			
	CD22 knockout			
	CD45 E613R point mutation			
	B cells deficient in all Src-family kinases (triple knockout)			
	FcγRIIB knockout (inhibitory signaling molecule)		*FCGR2A*	Lupus
Co-stimulatory molecules	CTLA-4 knockout (blocks inhibitory signal)	Lymphocyte infiltration into organs		
	PD-1 knockout (blocks inhibitory signal)	Lupus-like		
	BAFF overexpression (transgenic mouse)			
Apoptosis	Fas knockout (*lpr*)	Lupus-like with lymphocyte infiltrates	*FAS* and *FASL* mutations (ALPS)	Lupus-like with lymphocyte infiltrates
	FasL knockout (*gld*)			
	Bcl-2 overexpression (transgenic mouse)	Lupus-like		
	Pten heterozygous deficiency			
T_{reg} development/ function	*scurfy* mouse	Multi-organ autoimmunity	*FOXP3*	IPEX
	foxp3 knockout			

Fig. 15.31 Categories of genetic defects that lead to autoimmune syndromes. Many genes have been identified in which mutations predispose to autoimmunity in humans and animal models. These are best understood by the type of process affected by the genetic defect. A list of such genes is given here, organized by process (see the text for further discussion). In some cases, the same gene has been identified in mice and humans. In other cases, different genes affecting the same mechanism are implicated in mice and humans. The smaller number of human genes identified so far undoubtedly reflects the difficulty of identifying the genes responsible in outbred human populations.

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genetic markers, duplications or deletions, or **single-nucleotide polymorphisms** (**SNPs**), positions in the genome that differ by a single base between individuals. These studies have supported the concept that genetic susceptibility to autoimmune disease in humans is typically due to a combination of susceptibility alleles at multiple loci. For example, in large association studies looking at candidate susceptibility genes in humans, several of the commonest autoimmune diseases, including type 1 diabetes, Graves' disease, Hashimoto's thyroiditis, Addison's disease, rheumatoid arthritis, and multiple sclerosis, show genetic association with the *CTLA4* locus on chromosome 2. The cell-surface protein CTLA-4 is produced by activated T cells and is an inhibitory receptor for B7 co-stimulatory molecules (see Section 9-13). The effects of genetic variation in *CTLA4* on susceptibility to type 1 diabetes have been studied in mice. *CTLA4* is located on mouse chromosome 1 in a cluster with the genes for the other co-stimulatory receptors, CD28 and ICOS. When this genetic region in the diabetes-susceptible NOD mouse strain was replaced with the same region from the autoimmune-resistant B10 strain, it conferred diabetes resistance on the NOD mice. It seems that genetic variation in the splicing of the *CTLA4* mRNA may contribute to the difference in susceptibility. Splice variants of CTLA-4 that lack a portion essential for binding to its ligands B7.1 and B7.2 were still resistant to activation, and there was increased expression of this variant in the memory and regulatory T cells of diabetes-resistant mice.

15-20 Many genes that predispose to autoimmunity fall into categories that affect one or more of the mechanisms of tolerance.

The genes identified as predisposing to autoimmunity can be classified as follows: genes that affect autoantigen availability and clearance; those that affect apoptosis; those that affect signaling thresholds; those involved in cytokine gene expression or signaling; those affecting the expression of co-stimulatory molecules or their receptors; and those that affect the development or function of regulatory T cells (see Figs 15.30 and 15.31).

Genes that control antigen availability and clearance are important both centrally in the thymus, where they act to make self proteins available for inducing tolerance in developing lymphocytes, and in the periphery, where they control how self molecules are made available in an immunogenic form to peripheral lymphocytes. In the periphery, a hereditary deficiency of some complement proteins, specifically those for C1q, C2, and C4, is strongly associated with the development of SLE in humans. C1q, C2, and C4 are early components in the classical complement pathway, which is important in antibody-mediated clearance of apoptotic cells and immune complexes (see Chapter 2). If apoptotic cells and immune complexes are not cleared, the chance that their antigens will activate low-affinity self-reactive lymphocytes in the periphery is increased. Genes that control apoptosis, such as *Fas*, are important in regulating the duration and vigor of immune responses. Failure to regulate immune responses properly could cause excessive destruction of self tissues, releasing autoantigens. In addition, because clonal deletion and anergy are not absolute, immune responses can include some self-reactive cells. As long as their numbers are limited by apoptotic mechanisms, they may not be sufficient to cause autoimmune disease, but they could cause a problem if apoptosis is not properly regulated.

Perhaps the largest category of mutations associated with autoimmunity comprises those associated with signals that control lymphocyte activation. One subset contains mutations that inactivate negative regulators of lymphocyte activation and thus lead to the hyperproliferation of lymphocytes and exaggerated immune responses. These include mutations in CTLA-4 (as discussed

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in Section 15-19), in inhibitory Fc receptors, and in inhibitory receptors containing ITIMs, such as CD22 on B cells, which is a negative regulator of B-cell receptor signaling. Another subset contains mutations in proteins involved in signal transduction through the antigen receptor itself. Adjusting thresholds in either direction, to make signaling more or less sensitive, can result in autoimmunity, depending on the situation. A decrease in sensitivity in the thymus, for example, can lead to a failure of negative selection and thereby to autoreactivity in the periphery. In contrast, increasing receptor sensitivity in the periphery can lead to greater and prolonged activation, again resulting in an exaggerated immune response with the side effect of autoimmunity. Additionally, mutations that affect the expression of genes for cytokines and co-stimulatory molecules have been linked to autoimmunity. A final subset of mutations comprises those that predispose to autoimmunity by compromising regulatory T-cell development or function, as exemplified by the FoxP3 mutations that give rise to the IPEX syndrome (see Section 15-21).

15-21 A defect in a single gene can cause autoimmune disease.

Predisposition to most of the common autoimmune diseases is due to the combined effects of multiple genes, but there are very few known monogenic autoimmune diseases. In these, possession of the predisposing allele confers a very high risk of disease on the individual, but the overall impact on the population is minimal because these variants are rare (Fig. 15.32). The existence of monogenic autoimmune disease was first observed in mutant mice, in which the inheritance of an autoimmune syndrome followed a pattern consistent with a single-gene defect. Autoimmune disease alleles are usually recessive or X-linked. For example, the disease APECED, discussed in Section 15-3, is a recessive autoimmune disease caused by a defect in the gene *AIRE*.

Two monogenic autoimmune syndromes have been linked to defects in regulatory T cells. The X-linked recessive autoimmune syndrome **IPEX** (immune dysregulation, polyendocrinopathy, enteropathy, X-linked disease) is typically caused by missense mutations in the gene for the transcription factor FoxP3, which is a key factor in the differentiation of some types of T_{reg} cells (see Section 9-18). Also known as XLAAD (X-linked autoimmunity-allergic dysregulation syndrome), this disease is characterized by severe allergic inflammation, autoimmune polyendocrinopathy, secretory diarrhea, hemolytic

Fig. 15.32 Single-gene traits associated with autoimmunity. Listed are examples of monogenic disorders that cause autoimmunity in humans. Mice with targeted deletions (knockout) or spontaneous mutations (for example *lpr/lpr*) in homologous genes have similar disease characteristics and are useful models for the study of the pathogenic basis for these disorders. APECED, autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy; APS-1, autoimmune polyglandular syndrome 1; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome; ALPS, autoimmune lymphoproliferative syndrome. The *lpr* mutation in mice affects the gene for Fas, whereas the *gld* mutation affects the gene for FasL. Reprinted from J.D. Rioux and A.K. Abbas: *Nature* 435:584–589, © 2005. With permission from Macmillan Publishers Ltd.

Single-gene traits associated with autoimmunity

Gene	Human disease	Mouse mutant or knockout	Mechanism of autoimmunity
AIRE	APECED (APS-1)	Knockout	Decreased expression of self antigens in the thymus, resulting in defective negative selection of self-reactive T cells
CTLA4	Association with Graves' disease, type 1 diabetes and others	Knockout	Failure of T-cell anergy and reduced activation threshold of self-reactive T cells
FOXP3	IPEX	Knockout and mutation (*scurfy*)	Decreased function of CD4 CD25 regulatory T cells
FAS	ALPS	*lpr/lpr;gld/gld* mutants	Failure of apoptotic death of self-reactive B and T cells
C1q	SLE	Knockout	Defective clearance of immune complexes and apoptotic cells

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Immune Dysregulation, Polyendocrinopathy, Enteropathy X-linked Disease (IPEX)

anemia, and thrombocytopenia, and usually leads to early death. Despite mutation of the *FOXP3* gene, the numbers of CD4 CD25 T_{reg} cells in the blood of individuals with IPEX were comparable with those in healthy individuals; however, the suppressive function normally displayed by cells with this phenotype was reduced. A spontaneous frameshift mutation in the mouse *Foxp3* gene (the *scurfy* mutation) that results in loss of the DNA-binding, forkhead domain of FoxP3, or the knockout of *Foxp3* lead to an analogous systemic autoimmune disease, in this case associated with the absence of CD4 CD25 T_{reg} cells.

A second instance of autoimmunity resulting from a genetic defect in T_{reg}-cell function has been identified in a single patient with a deficiency of CD25 as a result of a deletion in *CD25* and impaired peripheral tolerance. This patient suffered from multiple immunological deficiencies and autoimmune diseases and was highly susceptible to infections. These findings further confirm the important roles of CD25 CD4 T_{reg} cells in the regulation of the immune system.

An interesting case of a monogenic autoimmune disease is the systemic autoimmune syndrome caused by mutations in the gene for Fas, which is called **autoimmune lymphoproliferative syndrome** (**ALPS**) in humans. Fas is normally present on the surface of activated T and B cells, and when ligated by Fas ligand it signals the Fas-bearing cell to undergo apoptosis (see Section 9-25). In this way it functions to limit the extent of immune responses. Mutations that eliminate or inactivate Fas lead to a massive accumulation of lymphocytes, especially T cells, and in mice the production of large quantities of pathogenic autoantibodies. The resulting disease resembles SLE, although typical SLE in humans has not been associated with mutations in Fas. A mutation leading to this autoimmune syndrome was first observed in the mouse strain MRL and dubbed *lpr*, for lymphoproliferation; it was subsequently identified as a mutation in the *Fas* gene. Researchers studying a group of human patients with the rare autoimmune lymphoproliferative syndrome, a syndrome similar to that in the MRL/*lpr* mice, identified and cloned the mutant gene responsible for most of these cases, which also turned out to be *FAS* (see Fig. 15.32).



Autoimmune Lymphoproliferative Syndrome (ALPS)

Autoimmune diseases caused by single genes are not common. They are nonetheless of great interest, as the mutations that cause them identify some of the important pathways that normally prevent the development of autoimmune responses.

15-22 MHC genes have an important role in controlling susceptibility to autoimmune disease.

Among all the genetic loci that could contribute to autoimmunity, susceptibility to autoimmune disease has so far been most consistently associated with MHC genotype. Human autoimmune diseases that show associations with HLA (MHC) type are shown in Fig. 15.33. For most of these diseases, susceptibility is linked most strongly with MHC class II alleles, and thus with CD4 T cells, although in some cases there are strong associations with particular MHC class I alleles, implicating CD8 T cells. In some cases, class III alleles such as those for TNF-α or complement protein have been associated with disease. The development of experimental diabetes or arthritis in transgenic mice expressing specific human HLA antigens strongly suggests that particular MHC alleles can confer susceptibility to disease.

The association of MHC genotype with disease is assessed initially by comparing the frequency of different alleles in patients with their frequency in the normal population. For type 1 diabetes, this approach originally demonstrated an association with the HLA-DR3 and HLA-DR4 alleles identified by serotyping (Fig. 15.34). Such studies also showed that the MHC class II allele

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Associations of HLA serotype with susceptibility to autoimmune disease			
Disease	**HLA allele**	**Relative risk**	**Sex ratio (♀:♂)**
Ankylosing spondylitis	B27	87.4	0.3
Acute anterior uveitis	B27	10	<0.5
Goodpasture's syndrome	DR2	15.9	~1
Multiple sclerosis	DR2	4.8	10
Graves' disease	DR3	3.7	4–5
Myasthenia gravis	DR3	2.5	~1
Systemic lupus erythematosus	DR3	5.8	10–20
Type 1 (insulin-dependent) diabetes mellitus	DR3/DR4 heterozygote	~25	~1
Rheumatoid arthritis	DR4	4.2	3
Pemphigus vulgaris	DR4	14.4	~1
Hashimoto's thyroiditis	DR5	3.2	4–5

Fig. 15.33 Associations of HLA serotype and sex with susceptibility to autoimmune disease. The 'relative risk' for an HLA allele in an autoimmune disease is calculated by comparing the observed number of patients carrying the HLA allele with the number that would be expected, given the prevalence of the HLA allele in the general population. For type 1 insulin-dependent diabetes mellitus, the association is in fact with the HLA-DQ gene, which is tightly linked to the DR genes but is not detectable by serotyping. Some diseases show a significant bias in the sex ratio; this is taken to imply that sex hormones are involved in pathogenesis. Consistent with this, the difference in the sex ratio in these diseases is greatest between the menarche and the menopause, when levels of such hormones are highest.

HLA-DR2 has a dominant protective effect: individuals carrying HLA-DR2, even in association with one of the susceptibility alleles, rarely develop diabetes. Another way of determining whether MHC genes are important in autoimmune disease is to study the families of affected patients; it has been shown that two siblings affected with the same autoimmune disease are far more likely than expected to share the same MHC haplotypes (Fig. 15.35). As HLA genotyping has become more exact through the DNA sequencing of HLA alleles, disease associations that were originally discovered through HLA serotyping have been defined more precisely. For example, the association between type 1 diabetes and the DR3 and DR4 alleles is now known to be due to their tight genetic linkage to DQβ alleles that actually confer susceptibility to the disease. Indeed, susceptibility is most closely associated with polymorphisms at a particular position in the DQβ amino acid sequence. The most common DQβ amino acid sequence has an aspartic acid residue at position 57 that is able to form a salt bridge across the end of the peptide-binding cleft of the DQ molecule. In contrast, patients with diabetes in Caucasoid populations mostly have valine, serine, or alanine at that position, and thus make DQ molecules that lack this salt bridge (Fig. 15.36). The NOD strain of mice, which

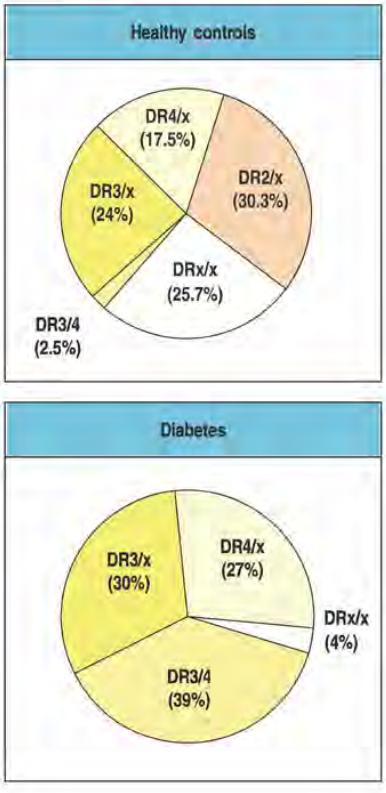


Fig. 15.34 Population studies show association of susceptibility to type 1 diabetes with HLA genotype. The HLA genotypes (determined by serotyping) of patients with diabetes (lower panel) are not representative of those found in the general population (upper panel). Almost all patients with diabetes express HLA-DR3 and/or HLA-DR4, and HLA-DR3/DR4 heterozygosity is greatly over-represented in diabetics compared with controls. These alleles are linked tightly to HLA-DQ alleles that confer susceptibility to type 1 diabetes. By contrast, HLA-DR2 protects against the development of diabetes and is found only extremely rarely in patients with diabetes. The small letter x represents any allele other than DR2, DR3, or DR4.

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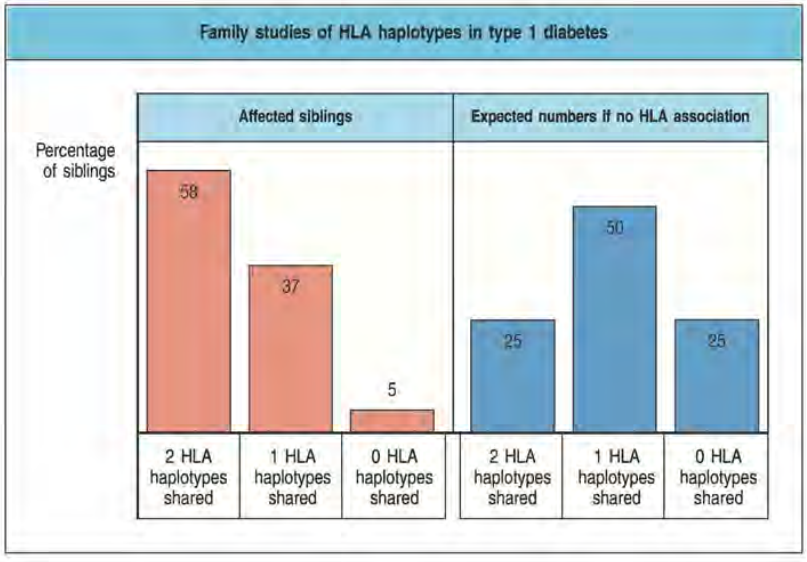


Fig. 15.35 Family studies show strong linkage of susceptibility to type 1 diabetes with HLA genotype. In families in which two or more siblings have type 1 diabetes, it is possible to compare the HLA genotypes of affected siblings. Affected siblings share two HLA haplotypes much more frequently than would be expected if the HLA genotype did not influence disease susceptibility.

develops spontaneous diabetes, also has a serine residue at that position in the homologous mouse MHC class II molecule, known as I-A^{g7}.

The association of MHC genotype with autoimmune disease is not surprising, because autoimmune responses involve T cells, and the ability of T cells to respond to a particular antigen depends on MHC genotype. Thus, the associations can be explained by a simple model in which susceptibility to an autoimmune disease is determined by differences in the ability of different allelic variants of MHC molecules to present autoantigenic peptides to autoreactive T cells. This would be consistent with what we know of T-cell involvement in particular diseases. In diabetes, for example, there are associations with both MHC class I and MHC class II alleles, and this is consistent with the finding that both CD8 and CD4 T cells, which respond to antigens presented by MHC class I and MHC class II molecules, respectively, mediate the autoimmune response.

An alternative hypothesis for the association between MHC genotype and susceptibility to autoimmune diseases emphasizes the role of MHC alleles in shaping the T-cell receptor repertoire (see Chapter 8). This hypothesis proposes that self peptides associated with certain MHC molecules may drive the positive selection of developing thymocytes that are specific for particular autoantigens. Such autoantigenic peptides might be expressed at too low a level or bind too poorly to self MHC molecules to drive negative selection in

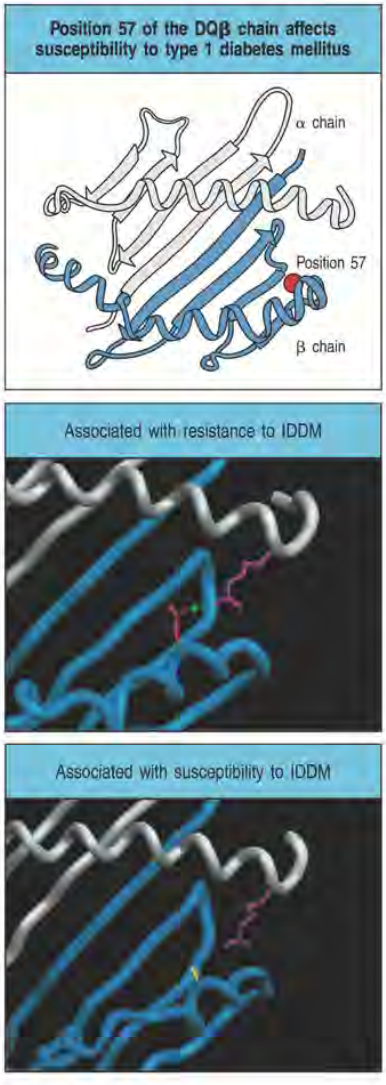


Fig. 15.36 Amino acid changes in the sequence of an MHC class II protein correlate with susceptibility to and protection from diabetes. The HLA-DQβ_1 chain contains an aspartic acid (Asp) residue at position 57 in most people; in Caucasoid populations, patients with type 1 diabetes more often have valine, serine, or alanine at this position instead, as well as other differences. Asp 57, shown in red on the backbone structure of the DQβ chain, forms a salt bridge (shown in green in the center panel) to an arginine residue (shown in pink) in the adjacent a chain (gray). The change to an uncharged residue (for example alanine, shown in yellow in the bottom panel) disrupts this salt bridge, altering the stability of the DQ molecule. The non-obese diabetic (NOD) strain of mice, which develops spontaneous diabetes, shows a similar substitution of serine for aspartic acid at position 57 of the homologous I-Aβ chain, and NOD mice transgenic for β chains with Asp 57 have a marked reduction in diabetes incidence. Courtesy of C. Thorpe.

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the thymus, but might be present at a sufficient level or bind strongly enough to drive positive selection. This hypothesis is supported by observations that I-A^{g7}, the disease-associated MHC class II molecule in NOD mice, binds many peptides very poorly and may therefore be less effective in driving intrathymic negative selection of T cells that bind self peptides.

15-23 Genetic variants that impair innate immune responses can predispose to T cell-mediated chronic inflammatory disease.

As noted earlier in this chapter, a common chronic inflammatory disease is **Crohn's disease**, an intestinal disorder of the type known generally as inflammatory bowel disease, or IBD. The other main form of IBD is ulcerative colitis. Crohn's disease results from an abnormal over-responsiveness of CD4 T cells to antigens of the normal commensal gut microbiota, as opposed to true self antigens. Abnormal hyperactivity of T_H1 and T_H17 cells is thought to be pathogenic in this disease. This results from a failure of mucosal innate immune mechanisms to sequester luminal bacteria from the adaptive immune system, or is caused by intrinsic defects in cells of the adaptive immune system that result in heightened effector CD4 T-cell responses or failure of homeostatic T_{reg}-cell activity to suppress their development (Fig. 15.37). Patients with Crohn's disease have episodes of severe inflammation that commonly affect the terminal ileum, with or without involvement of the colon—hence the alternative name of regional ileitis for this disease—but any part of the gastrointestinal tract can be involved. The disease is characterized by a chronic inflammation of the mucosa and submucosa of the intestine that includes the prominent development of granulomatous lesions (Fig. 15.38) similar to those seen in the type IV hypersensitivity responses discussed in Section 14-17. Genetic analysis of patients with Crohn's disease and their families has identified a growing list of disease-susceptibility genes. One of the earliest to be identified was *NOD2* (also known as *CARD15*), which is expressed predominantly in monocytes, dendritic cells, and the Paneth cells of the small intestine, and which is involved in recognition of microbial antigens as part of the innate immune response (see Section 3-8). Mutations and uncommon polymorphic variants of the NOD2 protein are strongly associated with the


Movie 15.1


Crohn's Disease

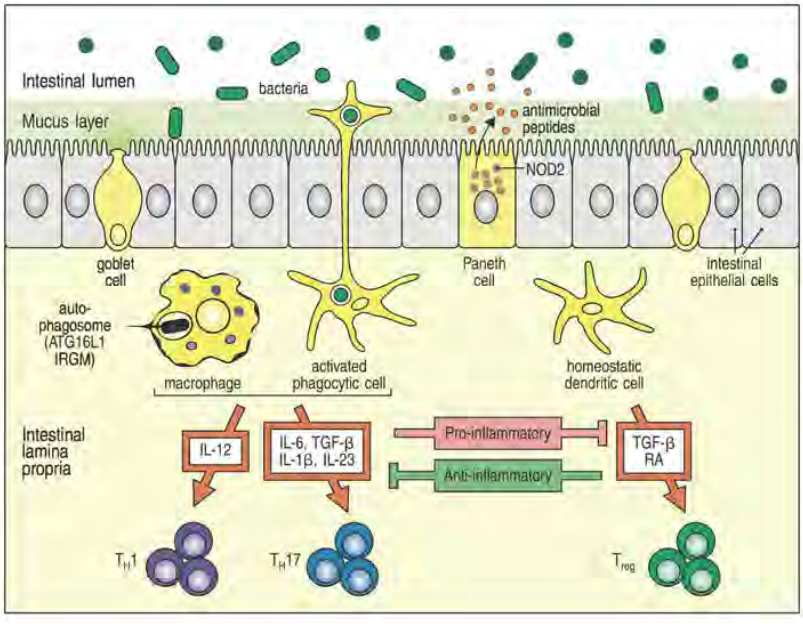


Fig. 15.37 Crohn's disease results from a breakdown of the normal homeostatic mechanisms that limit inflammatory responses to the gut microbiota. The innate and adaptive immune systems normally cooperate to limit inflammatory responses to intestinal bacteria through a combination of mechanisms: a mucus layer produced by goblet cells; tight junctions between the intestinal epithelial cells; antimicrobial peptides released from epithelial cells and Paneth cells; and induction of T_{reg} cells that inhibit effector CD4 T-cell development and promote the production of IgA antibodies that are transported into the intestinal lumen, where they inhibit translocation of intestinal bacteria (not shown). In individuals with impaired homeostatic mechanisms, dysregulated T_H1- and T_H17-cell responses to the intestinal microbiota can result, generating disease-causing chronic inflammation. Crohn's disease susceptibility genes of innate immunity include *NOD2* and the autophagy genes *ATG16L1* and *IRGM*. A major susceptibility gene that affects adaptive immune cells is *IL23R*, which is expressed by T_H17 cells.

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Fig. 15.38 Granulomatous inflammation in Crohn's disease. A section of bowel wall from a patient with Crohn's disease. The arrow marks a giant cell granuloma. There is a dense infiltrate of lymphocytes throughout the bowel submucosa. Photograph courtesy of H.T. Cook.

presence of Crohn's disease, with around 30% of patients carrying a loss-of-function mutation in *NOD2*. Mutations in the same gene are also the cause of a dominantly inherited granulomatous disease named **Blau syndrome**, in which granulomas typically develop in the skin, eyes, and joints. Whereas Crohn's disease results from a loss of function of NOD2, it is thought that Blau syndrome results from a gain of function.

NOD2 is an intracellular receptor for the muramyl dipeptide derived from bacterial peptidoglycan, and its stimulation leads to activation of the transcription factor NFκB and the induction of genes encoding pro-inflammatory cytokines and chemokines (see Section 3-8 and Fig. 12.19). In Paneth cells, which are specialized intestinal epithelial cells in the base of the small intestinal crypts, activation of NOD2 stimulates the release of granules containing antimicrobial proteins and peptides that contribute to the limitation of commensal bacteria to the intestinal lumen, away from cells of the adaptive immune system. Mutant forms of NOD2 that have lost this function limit the innate antibacterial response, thereby predisposing to heightened effector CD4 T-cell responses to the commensal microbiota that produce chronic intestinal inflammation (see Section 12-13).

In addition to NOD2, other deficiencies in innate immunity have been identified in patients with Crohn's disease, including defective CXCL8 production and defective neutrophil accumulation, which can synergize with defects in NOD2 to promote abnormal intestinal inflammation. Thus, compound defects in innate immunity and the regulation of inflammation may act synergistically to promote immunopathology in Crohn's disease. Genetic association studies have identified other susceptibility genes for Crohn's disease that may be linked to impaired innate immune functions. Two genes (*ATG161* and *IRGM*) that contribute to the process of autophagy have been linked to Crohn's disease, suggesting that other mechanisms that impair clearance of commensal bacteria might predispose to chronic intestinal inflammation. Autophagy, or the digestion of a cell's cytoplasm by its own lysosomes, is important in the turnover of damaged cellular organelles and proteins, and has a role in antigen processing and presentation (see Section 6-9), but is also thought to contribute to the clearance of some phagocytosed bacteria.

While defects in important pathways of the innate immune system contribute to Crohn's disease, genes that regulate the adaptive immune response have also been associated with susceptibility. Most notably, variants of the gene for the IL-23 receptor (*IL23R*) have been found that predispose to disease, consistent with heightened T_H17 responses found in diseased tissues. Collectively, the growing number of susceptibility genes that confer increased risk for Crohn's disease point to abnormal regulation of homeostatic innate and adaptive immune responses to the intestinal microbiota as a common factor.

15-24 External events can initiate autoimmunity.

The geographic distribution of autoimmune diseases reveals a heterogeneous distribution between continents, countries, and ethnic groups. For example, the incidence of disease in the Northern Hemisphere seems to decrease from north to south. This gradient is particularly prominent in diseases such as multiple sclerosis and type 1 diabetes in Europe, which have a higher incidence in the northern countries than in the Mediterranean regions. Several studies have also shown a reduced incidence of autoimmunity in developing countries compared with the more developed world.

There are numerous contributing factors to these geographic variations beside genetic susceptibility—socioeconomic status and diet seem to play a part. An example of how factors beside genetic background influence the onset of disease is the fact that even genetically identical mice develop autoimmunity

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at different rates and severity (see Fig. 15.29). In humans, exposure to infections and environmental toxins may be factors that help trigger autoimmunity. However, it should be noted that epidemiological and clinical studies over the past century have also shown a negative correlation between exposure to some types of infection in early life and the development of allergy and autoimmune diseases. This 'hygiene hypothesis' is discussed in detail in Section 14-4; it proposes that a lack of infection during childhood may affect the regulation of the immune system in later life, leading to a greater likelihood of allergic and autoimmune responses.

15-25 Infection can lead to autoimmune disease by providing an environment that promotes lymphocyte activation.

How might pathogens initiate or modulate autoimmunity? During an infection and the consequent immune response, the combination of the inflammatory mediators released from activated antigen-presenting cells and lymphocytes and the increased expression of co-stimulatory molecules can have effects on so-called bystander cells—lymphocytes that are not themselves specific for the antigens of the infectious agent. Self-reactive lymphocytes can become activated in these circumstances, particularly if tissue destruction by the infection leads to an increase in the availability of the self antigen (Fig. 15.39, first panel). Furthermore, pro-inflammatory cytokines, such as IL-1 and IL-6, impair the suppressive activity of regulatory T cells, allowing self-reactive naive T cells to become activated to differentiate into effector T cells that can initiate an autoimmune response.

In general, any infection will lead to an inflammatory response and recruitment of inflammatory cells to the site of the infection. The perpetuation, and even exacerbation, of autoimmune disease by viral or bacterial infections has been shown in experimental animal models. For example, the severity of type 1 diabetes in NOD mice is exacerbated by Coxsackie virus B4 infection, which leads to inflammation, tissue damage and the release of sequestered islet antigens, and the generation of autoreactive T cells.

We discussed earlier the ability of self ligands such as unmethylated CpG DNA sequences and RNA to directly activate ignorant autoreactive B cells via their TLRs and thus break tolerance to self (see Section 15-4). Microbial ligands for TLRs may also promote autoimmunity by stimulating dendritic cells and macrophages to produce large quantities of cytokines that cause local inflammation and help stimulate and maintain already activated autoreactive T cells and B cells. This mechanism might be relevant to the flare-ups of inflammation that follow infection in patients with autoimmune vasculitis associated with anti-neutrophil cytoplasmic antibodies.

One example of how exposure to TLR ligands can induce local inflammation derives from an animal model of arthritis in which injection of bacterial CpG DNA into the joints of healthy mice induces an aseptic arthritis characterized by macrophage infiltration. These macrophages express chemokine receptors on their surface and produce large amounts of CC chemokines, which promote leukocyte recruitment to the site of injection.

15-26 Cross-reactivity between foreign molecules on pathogens and self molecules can lead to anti-self responses and autoimmune disease.

Infection with certain pathogens is particularly associated with autoimmune sequelae. Some pathogens express protein or carbohydrate antigens that resemble host molecules, a phenomenon dubbed **molecular mimicry**. In such cases, antibodies produced against a pathogen epitope may cross-react with a self protein (see Fig. 15.39, second panel). Such structures do not

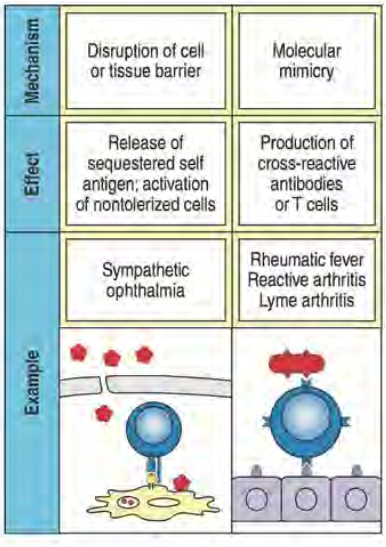


Fig. 15.39 Infectious agents could break self-tolerance in several different ways. Left panel: because some antigens are sequestered from the circulation, either behind a tissue barrier or within the cell, an infection that breaks cell and tissue barriers might expose hidden antigens. Right panel: molecular mimicry might result in infectious agents inducing either T- or B-cell responses that can cross-react with self antigens.

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necessarily have to be identical: it is sufficient if they are similar enough to be recognized by the same antibody. Molecular mimicry may also activate autoreactive naive or effector T cells if a processed peptide from a pathogen antigen is identical or similar to a host peptide, resulting in an attack on self tissues. A model system to demonstrate molecular mimicry has been generated by using transgenic mice that express a viral antigen in the pancreas. Normally, there is no response to this virus-derived 'self' antigen. But if the mice are infected with the virus that was the source of the transgenic antigen, they develop diabetes, because the virus activates T cells that are cross-reactive with the 'self' viral antigen and attack the pancreas (Fig. 15.40).

One might wonder why these self-reactive lymphocytes have not been deleted or inactivated by the usual mechanisms of self-tolerance. One reason, as discussed earlier in the chapter, is that lower-affinity self-reactive B and T cells are not removed efficiently and are present in the naive lymphocyte repertoire as ignorant lymphocytes (see Section 15-4). Second, the strong pro-inflammatory stimulus that accompanies an infection could be sufficient to activate even anergic T and B cells in the periphery, thus drawing into the response cells that would usually be silent. Third, pathogens may provide substantially higher local doses of the eliciting antigen in an immunogenic form, whereas normally it would be relatively unavailable to lymphocytes. Some examples of autoimmune syndromes thought to involve molecular mimicry are the rheumatic fever that sometimes follows streptococcal infection, and the reactive arthritis that can occur after enteric infection.

Autoimmmune Hemolytic Anemia

Once self-reactive lymphocytes have been activated by such a mechanism, their effector functions can destroy self tissues. Autoimmunity of this type is sometimes transient, and remits when the inciting pathogen is eliminated. This is the case in the autoimmune hemolytic anemia that follows mycoplasma infection, in which antibodies against the pathogen cross-react with an antigen on red blood cells, leading to hemolysis (see Section 15-13). The autoantibodies disappear when the patient recovers from the infection. Sometimes, however, the autoimmunity persists well beyond the initial infection. This is true in some cases of **rheumatic fever**, which occasionally follows sore throat or scarlet fever caused by *Streptococcus pyogenes*. The similarity of epitopes on streptococcal antigens to epitopes on some tissues leads to antibody-mediated, and possibly T cell-mediated, damage to a variety of tissues, including heart valves. Although rheumatic fever is often transient, especially with antibiotic treatment, it can sometimes become chronic. Similarly, Lyme

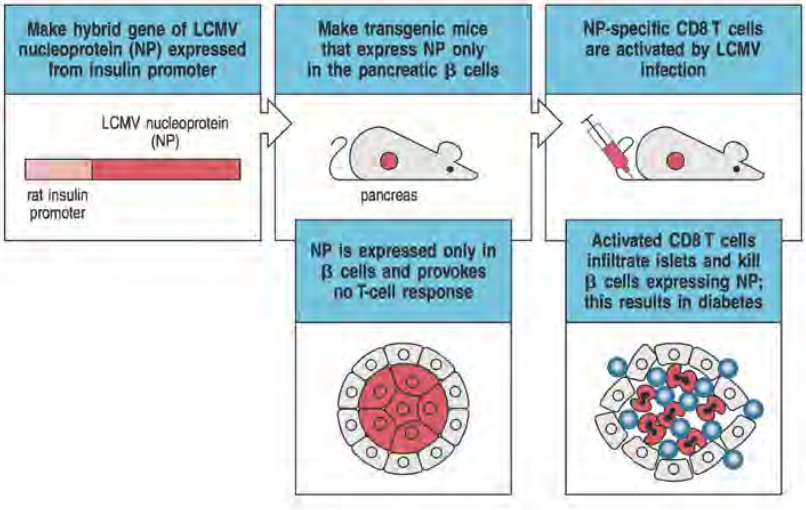


Fig. 15.40 Virus infection can break tolerance to a transgenic viral protein expressed in pancreatic β cells. Mice made transgenic for the lymphocytic choriomeningitis virus (LCMV) nucleoprotein under the control of the rat insulin promoter express the nucleoprotein in their pancreatic β cells but do not respond to this protein and therefore do not develop an autoimmune diabetes. However, if the transgenic mice are infected with LCMV, a potent antiviral cytotoxic T-cell response is elicited, and this kills the β cells, leading to diabetes. It is thought that infectious agents can sometimes elicit T-cell responses that cross-react with self peptides (a process known as molecular mimicry) and that this could cause autoimmune disease in a similar way.

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disease, an infection with the spirochete *Borrelia burgdorferi*, is followed by later-developing autoimmunity, causing so-called Lyme arthritis. In this case, the mechanism is not entirely clear, but it is likely to involve cross-reactivity of pathogen and host components, leading to a self-perpetuating autoimmune reaction.

15-27 Drugs and toxins can cause autoimmune syndromes.

Perhaps some of the clearest evidence of external causative agents in human autoimmunity comes from the effects of certain drugs, which elicit autoimmune reactions as side effects in a small proportion of patients. Procainamide, a drug used to treat heart arrhythmias, is particularly notable for inducing autoantibodies similar to those in SLE, although these are rarely pathogenic. Several drugs are associated with the development of autoimmune hemolytic anemia, in which autoantibodies against surface components of red blood cells attack and destroy these cells (see Section 15-13). Toxins in the environment can also cause autoimmunity. When heavy metals, such as gold or mercury, are administered to genetically susceptible strains of mice, a predictable autoimmune syndrome, including the production of autoantibodies, ensues. The extent to which heavy metals promote autoimmunity in humans is debatable, but the animal models clearly show that environmental factors such as toxins could have key roles in certain syndromes.

The mechanisms by which drugs and toxins cause autoimmunity are uncertain. For some drugs it is thought that they react chemically with self proteins and form derivatives that the immune system recognizes as foreign. The immune response to these haptenated self proteins can lead to inflammation, complement deposition, destruction of tissue, and finally immune responses to the original underivatized self proteins.

15-28 Random events may be required for the initiation of autoimmunity.

Although scientists and physicians would like to attribute the onset of 'spontaneous' diseases to some specific cause, this may not always be possible. There may not be one virus or bacterium, or even any understandable pattern of events that precedes the onset of autoimmune disease. The chance encounter in the peripheral lymphoid tissues of a few autoreactive B and T cells that can interact with each other, at just the moment when an infection is providing pro-inflammatory signals, may be all that is needed. This could be a rare event, and in a genetically resistant individual it could even be brought under control. But in a susceptible individual such events could be more frequent and/or more difficult to control.

Thus, the onset or incidence of autoimmunity can seem to be random. Genetic predisposition represents, in part, an increased chance of occurrence of this random event. This view, in turn, may explain why many autoimmune diseases appear in early adulthood or later, after enough time has elapsed to permit low-frequency random events to occur. It may also explain why after certain kinds of experimental aggressive therapies for these diseases, such as bone marrow transplantation or B-cell depletion, the disease eventually recurs after a long interval of remission.

Summary.

The specific causes of most autoimmune diseases are in most cases not known. Genetic risk factors including particular alleles of MHC class II molecules and other genes have been identified, but many individuals with genetic variants that predispose to a particular autoimmune disease do not get the disease.

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Epidemiological studies of genetically identical populations of animals have highlighted the role of environmental factors for the initiation of autoimmunity, but although environmental factors have at least as strong an influence on the outcome as genetics, they are even less well understood. Some toxins and drugs are known to cause autoimmune syndromes, but their role in the common varieties of autoimmune disease is unclear. Similarly, some autoimmune syndromes can follow viral or bacterial infections. Pathogens can promote autoimmunity by causing nonspecific inflammation and tissue damage. They can also sometimes elicit responses to self proteins if they express molecules that resemble self, a phenomenon known as molecular mimicry. Much more progress needs to be made to define environmental factors. It may be that there will not be a single, or even an identifiable, environmental factor that contributes to most diseases, and chance may have an important role in determining disease onset.

Responses to alloantigens and transplant rejection.

The transplantation of tissues to replace diseased organs is now an important medical therapy. In most cases, adaptive immune responses to the grafted tissues are the major impediment to successful transplantation. Rejection is caused by immune responses to alloantigens on the graft, which are proteins that vary from individual to individual within a species and are therefore perceived as foreign by the recipient. When tissues containing nucleated cells are transplanted, T-cell responses to the highly polymorphic MHC molecules almost always trigger a response against the grafted organ. Matching the MHC type of the donor and the recipient increases the success rate of grafts, but perfect matching is possible only when donor and recipient are related and, in these cases, genetic differences at other loci can still trigger rejection, although less severely. Nevertheless, advances in immunosuppression and transplantation medicine now mean that the precise matching of tissues for transplantation is no longer the major factor in graft survival. In blood transfusion, which was the earliest tissue transplant and is still the most common, MHC matching is not necessary for routine blood transfusions because red blood cells and platelets express only small amounts of MHC class I molecules and do not express MHC class II at all; thus, they are not usually targets for the T cells of the recipient. Antibodies made against platelet MHC class I can, however, be a problem when repeated transfusions of platelets are required. Blood must be matched for ABO and Rh blood group antigens to avoid the rapid destruction of mismatched red blood cells by antibodies in the recipient (see Appendix I, Section A-11). Because there are only four major ABO types and two Rh types, this is relatively easy. In this part of the chapter we examine the immune response to tissue grafts and also ask why such responses do not reject the one foreign tissue graft that is tolerated routinely—the mammalian fetus.

15-29 Graft rejection is an immunological response mediated primarily by T cells.

The basic rules of tissue grafting were first elucidated by skin transplantation between inbred strains of mice. Skin can be grafted with 100% success between different sites on the same animal or person (an **autograft**), or between genetically identical animals or people (a **syngeneic graft**). However, when skin is grafted between unrelated or **allogeneic** individuals (an **allograft**), the graft initially survives but is then rejected about 10–13 days after grafting (Fig. 15.41). This response is called an **acute rejection** and is quite

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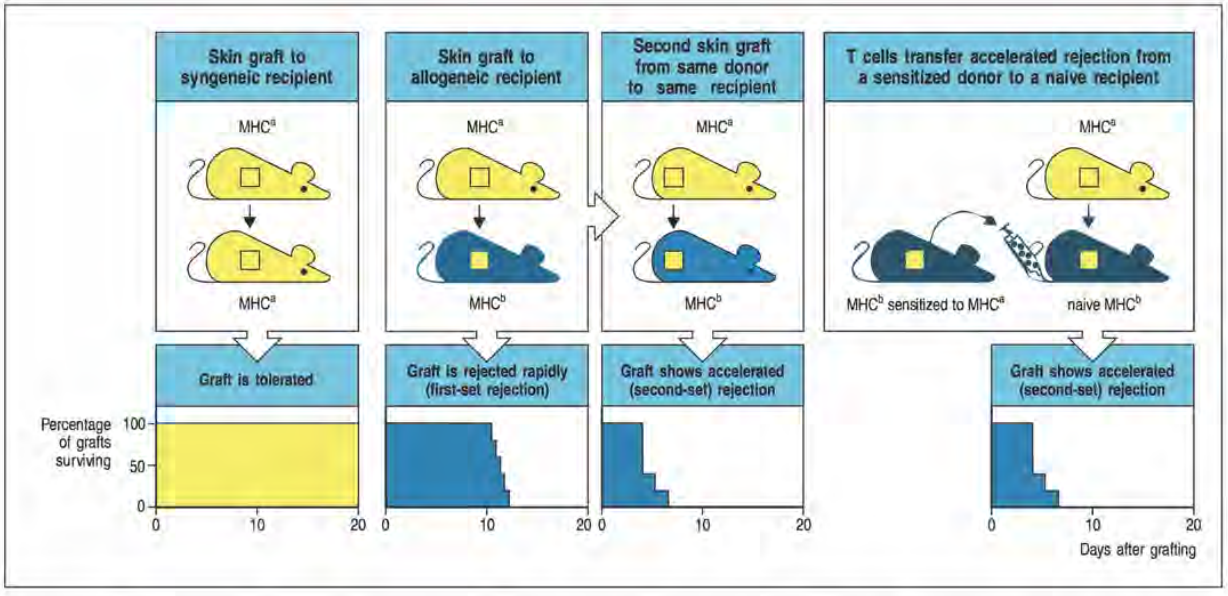


Fig. 15.41 Skin graft rejection is the result of a T cell-mediated anti-graft response. Grafts that are syngeneic are permanently accepted (first panels), but grafts differing at the MHC are rejected about 10–13 days after grafting (first-set rejection, second panels). When a mouse is grafted for a second time with skin from the same donor, it rejects the second graft faster (third panels). This is called a second-set rejection, and the accelerated response is MHC-specific; skin from a second donor of the same MHC type is rejected equally fast, whereas skin from an MHC-different donor is rejected in a first-set pattern (not shown). Naive mice that are given T cells from a sensitized donor behave as if they had already been grafted (final panels).

consistent. It depends on a T-cell response in the recipient, because skin grafted onto *nude* mice, which lack T cells, is not rejected. The ability to reject skin can be restored to *nude* mice by the adoptive transfer of normal T cells.

When a recipient that has previously rejected a graft is regrafted with skin from the same donor, the second graft is rejected more rapidly (6–8 days) in an **accelerated rejection** (see Fig. 15.41). Skin from a third-party donor grafted onto the same recipient at the same time does not show this faster response but follows a first-set rejection course. The rapid course of second-set rejection can also be transferred to normal or irradiated recipients by T cells from the initial recipient, showing that second-set rejection is caused by a memory-type immune response (see Chapter 11) from clonally expanded and primed T cells specific for the donor skin.

Immune responses are the major barrier to effective tissue transplantation, destroying grafted tissue by an adaptive immune response to its foreign proteins. These responses can be mediated by CD8 T cells, by CD4 T cells, or by both. Antibodies can also contribute to second-set rejection of tissue grafts.

15-30 Transplant rejection is caused primarily by the strong immune response to nonself MHC molecules.

Antigens that differ between members of the same species are known as **alloantigens**, and an immune response against such antigens is known as an **alloreactive** response. When donor and recipient differ at the MHC, an alloreactive immune response is directed at the nonself allogeneic MHC molecule or molecules present on the graft. In most tissues these will be predominantly MHC class I antigens. Once a recipient has rejected a graft of a particular MHC type, any further graft bearing the same nonself MHC molecule will be rapidly rejected in a second-set response. The frequency of T cells specific for any nonself MHC molecule is relatively high, making differences at MHC loci the most potent trigger of the rejection of initial grafts (see Section 6-14); indeed, the major histocompatibility complex was originally so named because of its central role in graft rejection.

Once it became clear that recognition of nonself MHC molecules was a major determinant of graft rejection, a considerable amount of effort was put into

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MHC matching between recipient and donor. Today, with advances in immunosuppression that have facilitated solid organ transplants across MHC barriers, MHC matching has become largely irrelevant for most types of allograft, although it remains important for bone marrow transplantation, for reasons that will be discussed later. Even a perfect match at the MHC locus, known as the HLA locus in humans, does not in itself prevent rejection reactions. Grafts between HLA-identical siblings will invariably incite a rejection reaction, albeit more slowly than an unmatched graft, unless donor and recipient are identical twins. This reaction is the result of differences between antigens from non-MHC proteins that also vary between individuals.

Thus, unless donor and recipient are identical twins, all graft recipients must be given immunosuppressive drugs chronically to prevent rejection. Indeed, the current success of clinical transplantation of solid organs is more the result of advances in immunosuppressive therapy, discussed in Chapter 16, than of improved tissue matching. The limited supply of cadaveric organs, coupled with the urgency of identifying a recipient once a donor organ becomes available, means that accurate matching of tissue types is achieved only rarely, with the notable exception of matched sibling donation of kidneys.

15-31 In MHC-identical grafts, rejection is caused by peptides from other alloantigens bound to graft MHC molecules.

When donor and recipient are identical at the MHC but differ at other genetic loci, graft rejection is not as rapid, but left unchecked it will still destroy the graft (Fig. 15.42). This is, for example, the reason that grafts between HLA-identical siblings would be rejected without immunosuppressive treatment. MHC class I and class II molecules bind and present a large selection of peptides derived from self proteins made in the cell, and if these proteins are polymorphic, then different peptides will be produced from them in different members of a species. Such proteins can be recognized as **minor histocompatibility antigens** (Fig. 15.43). One set of proteins that induce minor histocompatibility responses are encoded on the male-specific Y chromosome. Responses induced by these proteins are known collectively as H-Y. As these Y-chromosome-specific genes are not expressed in females, female anti-male minor histocompatibility responses occur; however, male anti-female responses are not seen, because both males and females express

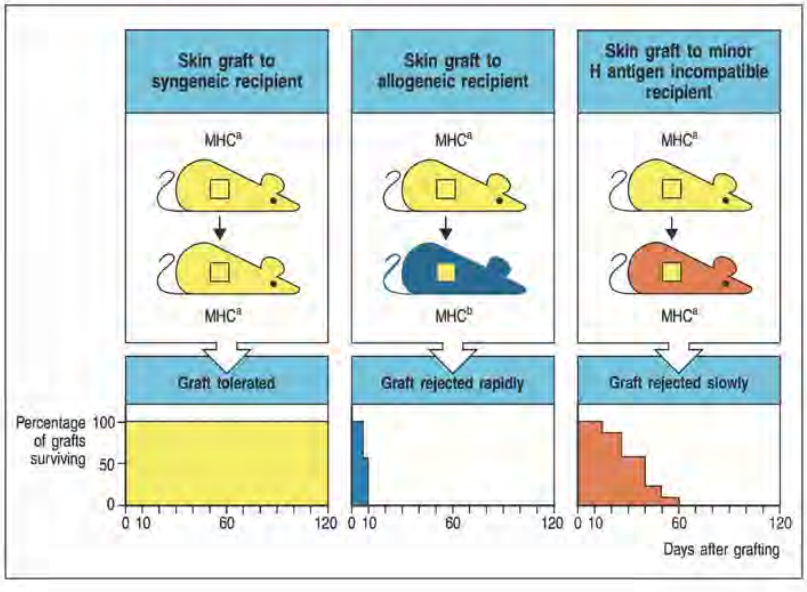


Fig. 15.42 Even complete matching at the MHC does not ensure graft survival. Although syngeneic grafts are not rejected (left panels), MHC-identical grafts from donors that differ at other loci (minor H antigen loci) are rejected (right panels), albeit more slowly than MHC-disparate grafts (center panels).

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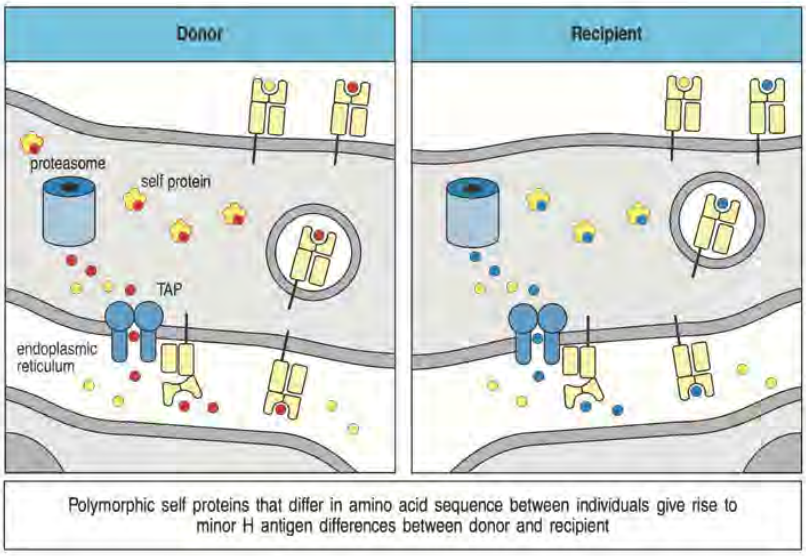


Fig. 15.43 Minor H antigens are peptides derived from polymorphic cellular proteins bound to MHC class I molecules. Self proteins are routinely digested by proteasomes within the cell's cytosol, and peptides derived from them are delivered to the endoplasmic reticulum, where they can bind to MHC class I molecules and be delivered to the cell surface. If a polymorphic protein differs between the graft donor (shown in red on the left) and the recipient (shown in blue on the right), it can give rise to an antigenic peptide (red on the donor cell) that can be recognized by the recipient's T cells as nonself and elicit an immune response. Such antigens are the minor H antigens.

X-chromosome genes. One H-Y antigen has been identified in mice and humans as peptides from a protein encoded by the Y-chromosome gene *Smcy*. An X-chromosome homolog of *Smcy*, called *Smcx*, does not contain these peptide sequences, which are therefore expressed uniquely in males. Most minor histocompatibility antigens are encoded by autosomal genes and their identity is largely unknown, although an increasing number have now been identified at the genetic level.

The response to minor histocompatibility antigens is in many ways analogous to the response to viral infection. However, whereas an antiviral response eliminates only infected cells, a large fraction of cells in the graft express minor histocompatibility antigens, and thus the graft is destroyed in the response against these antigens. Given the virtual certainty of mismatches in minor histocompatibility antigens between any two individuals, and the potency of the reactions they incite, it is understandable that successful transplantation requires the use of powerful immunosuppressive drugs.

15-32 There are two ways of presenting alloantigens on the transplanted donor organ to the recipient's T lymphocytes.

Before naive alloreactive T cells can develop into effector T cells that cause rejection, they must be activated by antigen-presenting cells that bear both the allogeneic MHC and co-stimulatory molecules. Organ grafts carry with them antigen-presenting cells of donor origin, sometimes called passenger leukocytes, and these are an important stimulus to alloreactivity. This route for sensitization of the recipient to a graft seems to involve donor antigen-presenting cells leaving the graft and migrating to secondary lymphoid tissues of the recipient, including the spleen and lymph nodes, where they can activate those host T cells that bear the corresponding T-cell receptors. Because the lymphatic drainage of solid organ allografts is interrupted by transplantation, migration of donor antigen-presenting cells occurs via the blood, not lymphatics. The activated alloreactive effector T cells can then circulate to the graft, which they attack directly (Fig. 15.44). This recognition pathway is known as **direct allorecognition** (Fig. 15.45, left panel). Indeed, if the grafted tissue is depleted of antigen-presenting cells by treatment with antibodies or by prolonged incubation, rejection occurs only after a much longer time.

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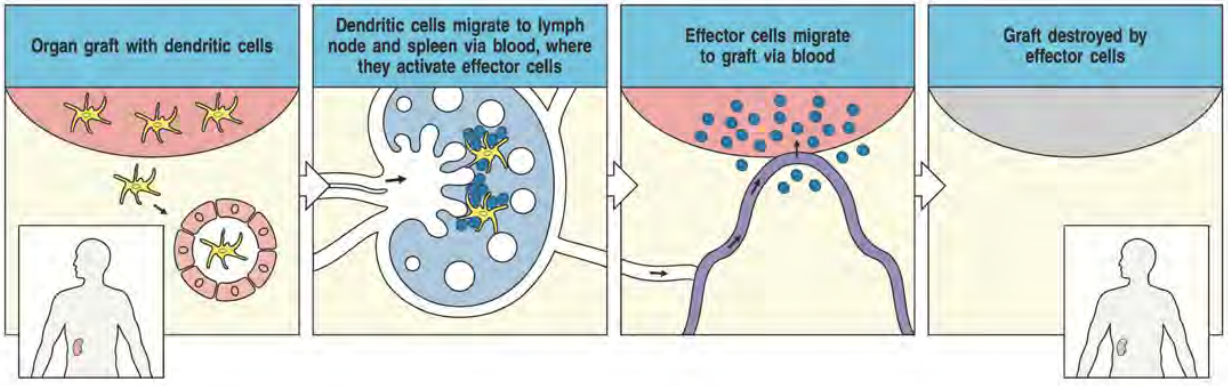


Fig. 15.44 The initiation of graft rejection normally involves the migration of donor antigen-presenting cells from the graft to local lymph nodes. The example of an organ graft is illustrated here, in which dendritic cells are the antigen-presenting cells. They display peptides from the graft on their surface. After traveling to the spleen or a lymph node, these antigen-presenting cells encounter recirculating naive T cells specific for graft antigens, and stimulate these T cells to divide. The resulting activated effector T cells migrate via the thoracic duct to the blood and home to the grafted tissue, which they rapidly destroy. Destruction is highly specific for donor-derived cells, suggesting that it is mediated by direct cytotoxicity and not by nonspecific inflammatory processes.

A second mechanism of allograft recognition leading to graft rejection is the uptake of allogeneic proteins by the recipient's own antigen-presenting cells and their presentation to T cells by self-MHC molecules. This is known as **indirect allorecognition** (Fig. 15.45, right panel). Peptides from both the foreign MHC molecules themselves and from the minor histocompatibility antigens can be presented by indirect allorecognition.

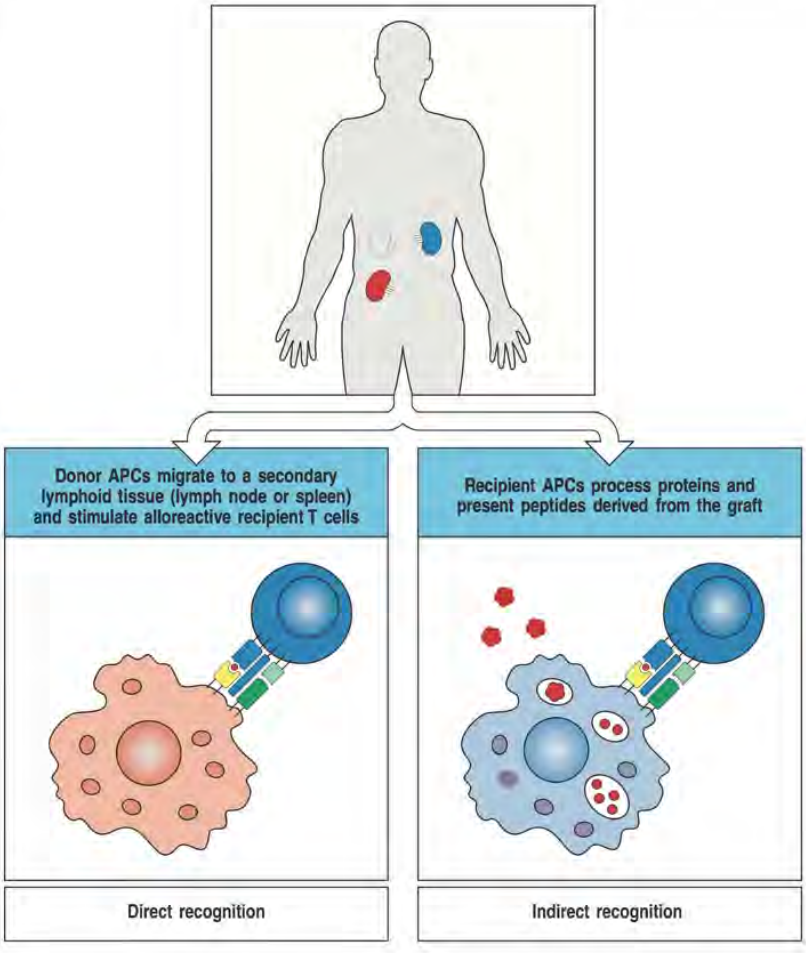


Fig. 15.45 Alloantigens in grafted organs are recognized in two different ways. Direct recognition of a grafted organ (red in upper panel) is by T cells whose receptors have specificity for the allogeneic MHC class I or class II molecule in combination with peptide. These alloreactive T cells are stimulated by donor antigen-presenting cells (APCs), which express both the allogeneic MHC molecule and co-stimulatory activity (lower left panel). Indirect recognition of the graft (lower right panel) involves T cells whose receptors are specific for allogeneic peptides derived from the grafted organ. Proteins from the graft (red) are taken up and processed by the recipient's antigen-presenting cells and are therefore presented by self (recipient) MHC class I or class II molecules.

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The relative contributions of direct and indirect allorecognition in graft rejection are not known. Direct allorecognition is thought to be largely responsible for acute rejection, especially when MHC mismatches mean that the frequency of directly alloreactive recipient T cells is high. Furthermore, a direct cytotoxic T-cell attack on graft cells can be made only by T cells that recognize the graft MHC molecules directly. Nonetheless, T cells with specificity for alloantigens presented on self MHC can contribute to graft rejection by activating macrophages, which cause tissue injury and fibrosis. T cells with indirect allospecificity are also likely to be important in the development of an antibody response to a graft. Antibodies produced against nonself antigens from another member of the same species are known as **alloantibodies**.

15-33 Antibodies that react with endothelium cause hyperacute graft rejection.

Antibody responses are an important potential cause of graft rejection. Preexisting alloantibodies against blood group antigens and polymorphic MHC antigens can cause the rapid rejection of transplanted organs in a complement-dependent reaction that can occur within minutes of transplantation. This type of reaction is known as **hyperacute graft rejection**. Most grafts that are transplanted routinely in clinical medicine are vascularized organ grafts linked directly to the recipient's circulation. In some cases the recipient might already have circulating antibodies against donor graft antigens. Antibodies of the ABO type can bind to all tissues, not just red blood cells. They are preformed and are relevant in all ABO-mismatched individuals. In addition, antibodies against other antigens can be produced in response to a previous transplant or a blood transfusion. All such preexisting antibodies can cause very rapid rejection of vascularized grafts because they react with antigens on the vascular endothelial cells of the graft and initiate the complement and blood clotting cascades. The vessels of the graft become blocked, or thrombosed, causing its immediate death. Such grafts become engorged and purple-colored from hemorrhaged blood, which becomes deoxygenated (Fig. 15.46). This problem can be avoided by ABO-matching as well as **cross-matching** donor and recipient. Cross-matching involves determining whether the recipient has antibodies that react with the white blood cells of the donor. If antibodies of this type are found, they have hitherto been considered a serious contraindication to transplantation of most solid organs, because in the absence of any treatment they lead to near-certain hyperacute rejection.

For reasons that are incompletely understood, some transplanted organs, particularly the liver, are less susceptible to this type of injury, and can be transplanted despite ABO incompatibilities. In addition, the presence of donor-specific MHC alloantibodies and a positive cross-match are no longer considered an absolute contraindication for transplantation, because the desensitization by treatment with intravenous immunoglobulin has been successful in a proportion of patients in whom antibodies against the donor tissue were already present.

A very similar problem prevents the routine use of animal organs—**xenografts**—in transplantation. If xenogeneic grafts could be used, it would circumvent the major limitation in organ replacement therapy, namely the

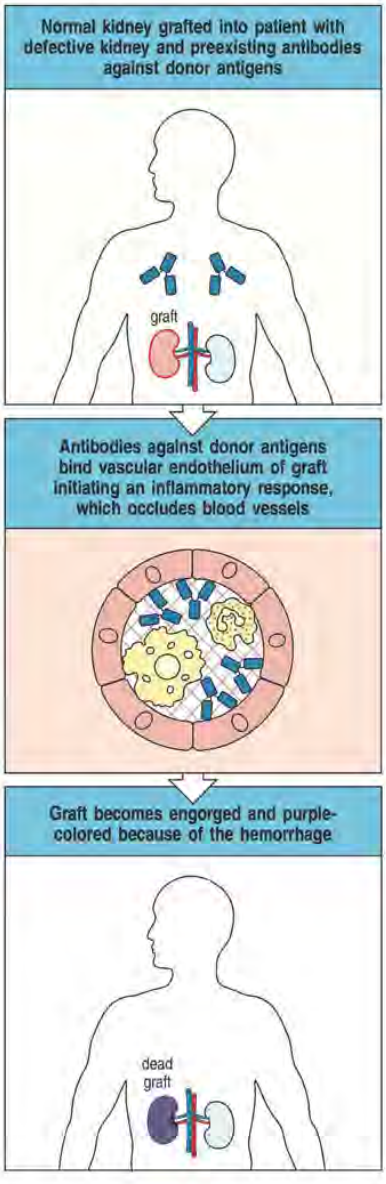


Fig. 15.46 Preexisting antibody against donor graft antigens can cause hyperacute graft rejection. In some cases, recipients already have antibodies against donor antigens, which are often blood group antigens. When the donor organ is grafted into such recipients, these antibodies bind to vascular endothelium in the graft, initiating the complement and clotting cascades. Blood vessels in the graft become obstructed by clots and leak, causing hemorrhage of blood into the graft. This becomes engorged and turns purple from the presence of deoxygenated blood.

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severe shortage of donor organs. Pigs have been suggested as a potential source of organs for xenografting, because they are of a similar size to humans and are easily farmed. Most humans and other primates have natural antibodies that react with a ubiquitous cell-surface carbohydrate antigen (α-Gal) of other mammalian species, including pigs. When pig xenografts are placed in humans, these antibodies trigger hyperacute rejection by binding to the endothelial cells of the graft and initiating the complement and clotting cascades. The problem of hyperacute rejection is exacerbated in xenografts because complement-regulatory proteins such as CD59, DAF (CD55), and MCP (CD46) (see Section 2-16) work less efficiently across a species barrier, and so pig regulatory proteins, for example, cannot protect the graft from attack by human complement.

A recent step toward xenotransplantation has been the development of transgenic pigs expressing human DAF as well as pigs that lack α-Gal. These approaches might one day reduce or eliminate hyperacute rejection in xenotransplantation. However, hyperacute rejection is only the first barrier faced by a xenotransplanted organ. The T lymphocyte-mediated graft rejection mechanisms might be extremely difficult to overcome with the immunosuppressive regimes currently available.

15-34 Late failure of transplanted organs is caused by chronic injury to the graft.

The success of modern immunosuppression means that about 90% of cadaveric kidney grafts are still functioning a year after transplantation. There has, however, been little improvement in rates of long-term graft survival: the half-life for functional survival of renal allografts remains about 8 years. Although traditionally the late failure of a transplanted organ has been termed **chronic rejection**, it is typically difficult to determine whether the cause of chronic allograft injury involves specific immune alloreactivity, nonimmune injury, or both.

The dominant pattern of chronic injury to transplanted organs is variable, depending on the tissue. A major component of late failure of vascularized transplanted organs is a chronic reaction called **chronic allograft vasculopathy**, which is a prominent cause of injury in heart and kidney allografts. This is characterized by concentric arteriosclerosis of graft blood vessels, which leads to hypoperfusion of the graft and its eventual fibrosis and atrophy. Multiple mechanisms may contribute to this form of vascular injury, including recurring acute rejection events, allospecific antibodies reactive to the vascular endothelium of the graft, or some forms of immunosuppressive therapy (for example calcineurin inhibitors, such as cyclosporin). In transplanted livers, chronic rejection is associated with loss of bile ducts, the so-called 'vanishing bile duct syndrome,' whereas in transplanted lungs, the major cause of late organ failure is accumulation of scar tissue in the smallest airways, or bronchioles, which is termed bronchiolitis obliterans. Alloreactive responses can occur months to years after transplantation, and may be associated with gradual loss of graft function that is hard to detect clinically.

Other important causes of chronic graft dysfunction include ischemia–reperfusion injury, which occurs at the time of grafting but may have late adverse effects on the grafted organ, viral infections that emerge as a result of immunosuppression, and recurrence of the same disease in the allograft that destroyed the original organ. Irrespective of etiology, chronic allograft injury is typically irreversible and progressive, ultimately leading to complete failure of allograft function.

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15-35 A variety of organs are transplanted routinely in clinical medicine.

Although the immune response makes organ transplantation difficult, there are few alternative therapies for organ failure. Three major advances have made it possible to use organ transplantation routinely in the clinic. First, surgical techniques for performing organ replacement have been advanced to the point at which they are now relatively routine in most major medical centers. Second, networks of transplantation centers have been organized to procure the few healthy organs that become available from cadaveric donors. Third, the use of powerful immunosuppressive drugs, especially cyclosporin A and tacrolimus (FK506) to inhibit T-cell activation (see Chapter 16), or blockade of IL-2 receptor signal with rapamycin (sirolimus), which provokes the apoptosis of allospecifically activated CD4 lymphocytes, has markedly increased graft survival rates. The different organs that are transplanted in the clinic are listed in Fig. 15.47. By far the most frequently transplanted solid organ is the kidney, the organ first successfully transplanted between identical twins in the 1950s. Transplantation of the cornea is even more frequent; this tissue is a special case because it is not vascularized, and corneal grafts between unrelated people are usually successful even without immunosuppression.

Many problems other than graft rejection are associated with organ transplantation. First, donor organs are difficult to obtain; this is especially a problem when the organ involved is a vital one, such as the heart or liver. Second, the disease that destroyed the patient's organ might also destroy the graft, as in the destruction of pancreatic β cells in autoimmune diabetes. Third, the immunosuppression required to prevent graft rejection increases the risk of cancer and infection. All of these problems need to be addressed before clinical transplantation can become commonplace. The problems most amenable to scientific solution are the development of more effective means of immunosuppression that prevent rejection with minimal impairment of more generalized immunity, the induction of graft-specific tolerance, and the development of xenografts as a practical solution to organ availability.

Tissue transplanted	No. of grafts in USA (2009)*	5-year graft survival
Kidney	17,683	81.4%[§]
Liver	6,320	68.3%
Heart	2,241	74.0%
Pancreas and pancreas/kidney	1,233	53.4%[†]
Lung	1,690	50.6%
Intestine	180	~48.4%
Cornea	~40,000	~70%
HSC transplants	15,000[‡]	40%/60%[‡]

Fig. 15.47 Tissues commonly transplanted in clinical medicine. The numbers of organ grafts performed in the United States in 2009 are shown. HSC, hematopoietic stem cells (includes bone marrow, peripheral blood HSCs, and cord blood transplants). *Number of grafts includes multiple organ grafts (for example kidney and pancreas, or heart and lung). For solid organs, 5-year survival is based on transplants performed between 2002 and 2007. Data from the United Network for Organ Sharing. [§]Kidney survival listed (81.4%) is for kidneys from living donors; 5-year survival for cadaveric donor transplants is 69.1%. [†]Pancreas survival listed (53.4%) is when transplanted alone; 5-year survival when transplanted with a kidney is 73.5%. [‡]Latest data available. They refer to allogeneic transplants only; survival depends on disease and is 40% for patients with acute and 60% for patients with chronic forms of myelogenous leukemia. All grafts except corneal grafts require long-term immunosuppression.

15-36 The converse of graft rejection is graft-versus-host disease.

Transplantation of hematopoietic stem cells (HSCs) enriched from peripheral blood, bone marrow, or fetal cord blood is a successful therapy for some tumors derived from bone marrow precursor cells, such as certain leukemias and lymphomas. By replacing genetically defective stem cells with normal donor ones, HSC transplantation can also be used to cure some primary immunodeficiency diseases (see Chapter 13) and other inherited diseases due to defective blood cells, such as the severe forms of thalassemia. In leukemia therapy, the recipient's bone marrow, the source of the leukemia, must first be destroyed by a combination of irradiation and aggressive cytotoxic chemotherapy. One of the major complications of allogeneic HSC transplantation is **graft-versus-host disease** (**GVHD**), in which mature donor T cells that contaminate preparations of HSCs recognize the tissues of the recipient as foreign, causing a severe inflammatory disease characterized by rashes, diarrhea, and liver disease. GVHD is particularly virulent when there is mismatch of MHC class I or class II antigens. Most transplants are therefore undertaken only when the donor and recipient are HLA-matched siblings or, less frequently, when there is an HLA-matched unrelated donor. As in organ transplantation, GVHD also occurs in the context of disparities between minor histocompatibility antigens, so immunosuppression must also be used in every HSC transplant.



Graft-Versus-Host Disease

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The presence of alloreactive donor T cells can easily be demonstrated experimentally by the **mixed lymphocyte reaction** (**MLR**), in which lymphocytes from a potential donor are mixed with irradiated lymphocytes from the potential recipient. If the donor lymphocytes contain naive T cells that recognize alloantigens on the recipient lymphocytes, they will respond by proliferating (Fig. 15.48). The MLR is sometimes used in the selection of donors for HSC transplants, when the lowest possible alloreactive response is essential. However, the limitation of the MLR in the selection of HSC donors is that the test does not accurately quantify alloreactive T cells. A more accurate test is a version of the limiting-dilution assay (see Appendix I, Section A-25), which precisely counts the frequency of alloreactive T cells.

Although GVHD is harmful to the recipient of a HSC transplant, it can have some beneficial effects that are crucial to the success of the therapy. Much of the therapeutic effect of HSC transplantation for leukemia can be due to a **graft-versus-leukemia effect**, in which the allogeneic preparations of HSCs contain donor T cells that recognize minor histocompatibility antigens or tumor-specific antigens expressed by the host leukemic cells, leading the donor cells to kill the leukemic cells. One of the treatment options for suppressing the development of GVHD is the elimination of mature T cells from the preparations of donor HSCs *in vitro* before transplantation, thereby removing alloreactive T cells. Those T cells that subsequently mature from the donor marrow *in vivo* in the recipient are tolerant to the recipient's antigens. Although the elimination of GVHD has benefits for the patient, there is an increased risk of leukemic relapse, which provides strong evidence in support of the graft-versus-leukemia effect.

Immunodeficiency is another complication of donor T-cell depletion. Because most of the recipient's T cells are destroyed by the combination of high-dose chemotherapy and irradiation used to treat the recipient before the transplant, donor T cells are the major source for reconstituting a mature T-cell repertoire after the transplant. This is particularly true in adults, who have poor residual thymic function. If too many T cells are depleted from the graft, therefore, transplant recipients experience, and often die from, numerous opportunistic infections. The need to balance the beneficial effects of

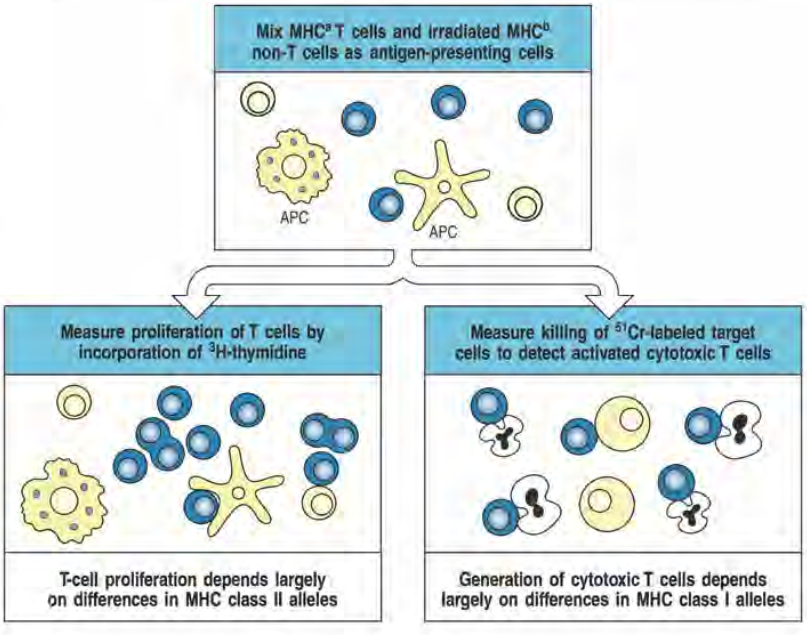


Fig. 15.48 The mixed lymphocyte reaction (MLR) can be used to detect histoincompatibility. Lymphocytes from the two individuals who are to be tested for compatibility are isolated from peripheral blood. The cells from one person (yellow), which will also contain antigen-presenting cells, are either irradiated or treated with mitomycin C; they will act as stimulator cells but cannot now respond by DNA synthesis and cell division to antigenic stimulation by the other person's cells. The cells from the two individuals are then mixed (upper panel). If the unirradiated lymphocytes (the responders, blue) contain alloreactive T cells, these will be stimulated to proliferate and differentiate to effector cells. Between 3 and 7 days after mixing, the culture is assessed for T-cell proliferation (lower left panel), which is mainly the result of CD4 T cells recognizing differences in MHC class II molecules, and for the generation of activated cytotoxic T cells (lower right panel), which respond to differences in MHC class I molecules.

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the graft-versus-leukemia effect and immunocompetence with the adverse effects of GVHD caused by donor T cells has spawned much research. One particularly promising approach is to prevent donor T cells from reacting with recipient antigens that they could meet shortly after the transplant. This is accomplished by depleting the recipient's antigen-presenting cells, chiefly dendritic cells (Fig. 15.49). Evidently, in this situation, the donor T cells are not activated during the initial inflammation that accompanies the transplant, and thereafter they do not promote GVHD. However, it is unclear whether there would be a graft-versus-leukemia effect in this context.

15-37 Regulatory T cells are involved in alloreactive immune responses.

As in all immune responses, regulatory T cells are now thought to have an important immunoregulatory role in the alloreactive immune responses involved in graft rejection. Experiments on the transplantation of allogeneic HSCs in mice have thrown some light on this question. Here, depletion of either CD4 CD25 T_{reg} cells in the recipient or of the same class of T_{reg} cells in the HSC graft before transplantation accelerated the onset of GVHD and subsequent death. In contrast, supplementing the graft with fresh CD4 CD25 T_{reg} cells or such cells that had been activated and expanded *ex vivo* delayed, or even prevented, death from GVHD. Similar observations have been made in experimental mouse models of solid organ transplantation, where the transfer of either naturally occurring or induced CD4 CD25 T_{reg} cells significantly delays allograft rejection. These experiments suggest that enriching or generating T_{reg} cells in preparations of donor HSCs might provide a possible therapy for GVHD in the future.

Another class of regulatory T cells, $CD8^+$ $CD28^-$ T_{reg} cells, have an anergic phenotype and they are thought to maintain T-cell tolerance indirectly by inhibiting the capacity of antigen-presenting cells to activate helper T cells. Cells of this type have been isolated from transplant patients. They can be distinguished from alloreactive CD8 cytotoxic T cells because they do not display cytotoxic activity against donor cells and express high levels of the inhibitory killer receptor CD94 (see Section 3-21). This finding suggests the possibility that $CD8^+$ $CD28^-$ T_{reg} cells interfere with the activation of antigen-presenting cells and have a role in the maintenance of transplant tolerance.

15-38 The fetus is an allograft that is tolerated repeatedly.

All of the transplants discussed so far are artifacts of modern medical technology. However, one 'foreign' tissue that is repeatedly grafted and repeatedly

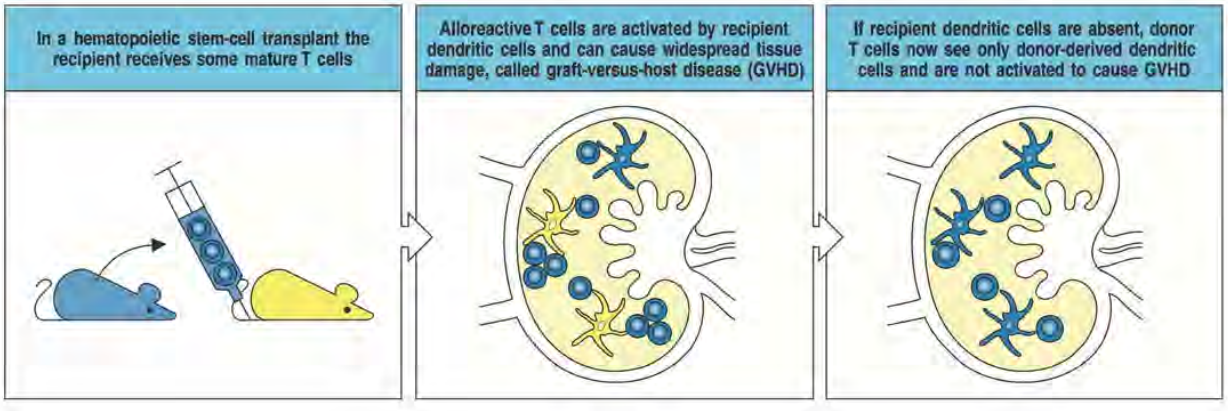


Fig. 15.49 Recipient type antigen-presenting cells are required for the efficient initiation of graft-versus-host disease (GVHD). T cells that accompany the hematopoietic stem cells from the donor (left panel) can recognize minor histocompatibility antigens of the recipient and start an immune response against the recipient's tissues. In stem-cell transplantation, minor antigens could be presented by either recipient- or donor-derived antigen-presenting cells, the latter deriving from the stem-cell graft and from precursor cells that differentiate after the transplant. Antigen-presenting cells are shown here as dendritic cells in a lymph node (middle panel). In mice, it has been possible to inactivate the host antigen-presenting cells by using gene knockouts. Such recipients are entirely resistant to GVHD mediated by donor CD8 T cells (right panel). Thus, cross-presentation of the recipient's minor histocompatibility antigens on donor dendritic cells is not sufficient to stimulate GVHD; those antigens endogenously synthesized and presented by the recipient's antigen-presenting cells are required to stimulate donor T cells. For this strategy to be useful for preventing GVHD in human patients, ways of depleting the recipient's antigen-presenting cells will be needed. These are the focus of research in several laboratories.

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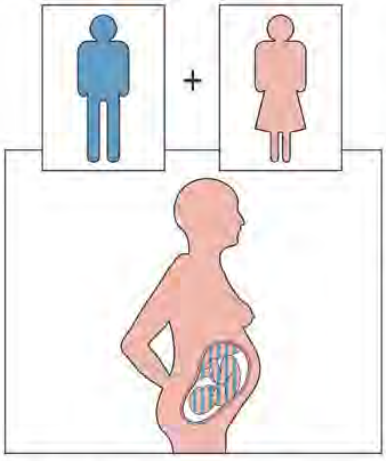


Fig. 15.50 The fetus is an allograft that is not rejected. Although the fetus carries MHC molecules derived from the father, and other foreign antigens, it is not rejected. Even when the mother bears several children to the same father, no sign of immunological rejection is seen.

tolerated is the mammalian fetus. The fetus carries paternal MHC and minor histocompatibility antigens that differ from those of the mother (Fig. 15.50), and yet a mother can successfully bear many children expressing the same nonself MHC proteins derived from the father. The mysterious lack of rejection of the fetus has puzzled generations of immunologists, and no comprehensive explanation has yet emerged. One problem is that acceptance of the fetal allograft is so much the norm that it is difficult to study the mechanism that prevents rejection; if the mechanism for rejecting the fetus is rarely activated, how can one analyze the mechanisms that control it?

Various hypotheses have been advanced to account for the tolerance shown to the fetus. It has been proposed that the fetus is simply not recognized as foreign. There is evidence against this hypothesis, because women who have borne several children usually make antibodies directed against the father's MHC proteins and red blood cell antigens; indeed, this is the best source of antibodies for human MHC typing. However, the placenta, which is a fetus-derived tissue, seems to sequester the fetus from the mother's T cells. The outer layer of the placenta, the interface between fetal and maternal tissues, is the trophoblast. This does not express classical MHC class I and class II proteins, making it resistant to recognition and attack by maternal T cells. Tissues lacking MHC class I expression are, however, vulnerable to attack by NK cells (see Section 3-21). The trophoblast might be protected from attack by NK cells by the expression of a nonclassical and minimally polymorphic HLA class I molecule—HLA-G. This protein has been shown to bind to the two major inhibitory NK receptors, KIR1 and KIR2, and to inhibit NK killing.

The placenta might also sequester the fetus from the mother's T cells by an active mechanism of nutrient depletion. The enzyme indoleamine 2,3-dioxygenase (IDO) is expressed at a high level by cells at the maternal–fetal interface. This enzyme catabolizes, and thereby depletes, the essential amino acid tryptophan at this site, and T cells starved of tryptophan show reduced responsiveness. Inhibition of IDO in pregnant mice, using the inhibitor 1-methyltryptophan, causes rapid rejection of allogeneic, but not syngeneic, fetuses. This supports the hypothesis that maternal T cells, alloreactive to paternal MHC proteins, might be held in check in the placenta by tryptophan depletion.

It is likely that fetal tolerance is a multifactorial process. The trophoblast does not act as an absolute barrier between mother and fetus, and fetal blood cells can cross the placenta and be detected in the maternal circulation, albeit in very low numbers. There is direct evidence from experiments in mice for specific T-cell tolerance against paternal MHC alloantigens. Pregnant female mice whose T cells bear a transgenic receptor specific for a paternal alloantigen showed reduced expression of this T-cell receptor during pregnancy. These same mice lost the ability to control the growth of an experimental tumor bearing the same paternal MHC alloantigen. After pregnancy, tumor growth was controlled and the level of the T-cell receptor increased. This experiment demonstrates that the maternal immune system must have been exposed to paternal MHC alloantigens and that the immune response to these antigens was temporarily suppressed.

Yet another factor that might contribute to maternal tolerance of the fetus is the secretion of cytokines at the maternal–fetal interface. Both the uterine epithelium and the trophoblast secrete cytokines, including TGF-β and IL-10. This combination of cytokines tends to suppress the development of effector T-cell responses (see Section 11-5). The induction or injection of cytokines such as IFN-γ and IL-12, which promote T_H1 responses in experimental animals, promotes fetal resorption, the equivalent of spontaneous abortion in humans. Finally, it is possible that regulatory T cells could have a role in suppressing responses to the fetus.

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The fetus is thus tolerated for two main reasons: it occupies a site protected by a nonimmunogenic tissue barrier, and it promotes a local immunosuppressive response in the mother. Several sites in the body, such as the eye, have these characteristics and allow the prolonged acceptance of foreign tissue grafts. They are usually called immunologically privileged sites (see Section 15-5).

Summary.

Clinical transplantation is now an everyday reality, its success built on MHC matching, immunosuppressive drugs, and technical skill. However, even accurate MHC matching does not prevent graft rejection; other genetic differences between host and donor can result in allogeneic proteins whose peptides are presented as minor histocompatibility antigens by MHC molecules on the grafted tissue, and responses to these can lead to rejection. Because we lack the ability to specifically suppress the response to the graft without compromising host defense, most transplants require generalized immunosuppression of the recipient. This can be toxic and increases the risk of cancer and infection. The fetus is a natural allograft that must be accepted—it almost always is—or the species will not survive. Tolerance to the fetus might hold the key to inducing specific tolerance to grafted tissues, or it might be a special case not applicable to organ replacement therapy.

Summary to Chapter 15.

Ideally, the effector functions of the immune system would be targeted only to foreign pathogens and never to self tissues. In practice, because foreign and self proteins are chemically similar, strict discrimination between self and nonself is impossible. Yet the immune system maintains tolerance to self tissues. This is accomplished by layers of regulation, all of which use surrogate markers to distinguish self from nonself, thus properly directing the immune response. When these regulatory mechanisms break down, autoimmune disease can result. Minor breaches of single regulatory barriers probably occur every day but are quelled by the effects of other regulatory layers: thus, tolerance operates at the level of the overall immune system. For disease to occur, multiple layers of tolerance have to be overcome and the effect needs to be chronic. These layers begin with central tolerance in the bone marrow and thymus, and include peripheral mechanisms such as anergy, cytokine deviation, and regulatory T cells. Sometimes immune responses do not occur simply because the antigens are not available, as in immune sequestration.

Perhaps because of selective pressure to mount effective immune responses to pathogens, the damping of immune responses to promote self-tolerance is limited and prone to failure. Genetic predisposition has an important role in determining which individuals will develop an autoimmune disease. The MHC region has an important effect in many diseases. There are many other genes that contribute to immune regulation and thus, when defective, can cause or predispose to autoimmune disease. Environmental forces also have a significant role, because even identical twins are not always both affected by the same autoimmune disease. Influences from the environment could include infections, toxins, and chance events.

When self-tolerance is broken and autoimmune disease ensues, the effector mechanisms are quite similar to those used in pathogen responses. Although the details vary from disease to disease, both antibody and T cells can be involved. Much has been learned about immune responses made to tissue antigens by examining the response to nonself transplanted organs and tissues; lessons learned in the study of graft rejection apply to autoimmunity

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and vice versa. Transplantation of solid organs and hematopoietic stem cells has brought on syndromes of rejection that are in many ways similar to autoimmune disease, but the targets are either major or minor histocompatibility antigens. The latter come from polymorphic genes. T cells are the main effectors in graft rejection and graft-versus-host disease.

For each of the undesirable categories of response discussed here (along with allergy, discussed in Chapter 14), the question is how to control the response without adversely affecting protective immunity to infection. The answer might lie in a more complete understanding of the regulation of the immune response, especially the suppressive mechanisms that seem to be important in tolerance. The deliberate control of the immune response is examined further in Chapter 16.

Questions.

15.1 *What is the evidence that genetic predisposition has an important role in autoimmune disease? Give two examples, and for each explain why the example implicates genetics.*

15.2 *(a) Discuss one compelling piece of evidence that environment has a role in the development of autoimmunity. (b) Name two potential environmental factors, and for one of them describe in more detail how it might work to incite autoimmunity.*

15.3 *There are several different mechanisms that initiate autoimmunity. Provide and briefly discuss an example each for four of these. Include both antibody-dependent and T-cell dependent mechanisms.*

15.4 *Discuss potential mechanisms for and differences between hyperacute, acute, and chronic rejection.*

15.5 *(a) Describe the ways in which the fetus is protected from immune attack by the mother. (b) Nevertheless, newborn babies of mothers with some types of autoimmunity show symptoms of the same autoimmune disease. How does this happen and what can be done to alleviate it? Not all autoimmune diseases will affect a baby in this way; explain why.*

15.6 *What is the role of TNF-α in rheumatoid arthritis? Which cells does it come from?*

15.7 *Discuss the mechanisms leading to the production of donor antigen-reactive antibody in an allograft recipient and how such antibody could lead to injury to the graft.*

15.8 *What would be the consequences of depleting T cells from an allogeneic bone marrow transplant for a patient with leukemia? What explanation can you give for this effect?*

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The Immunologist's Toolbox

APPENDIX I

Immunization.

Natural adaptive immune responses are normally directed at antigens borne by pathogenic microorganisms. The immune system can also be induced to respond to simple nonliving antigens, and experimental immunologists have focused on the responses to these simple antigens in developing our understanding of the immune response. The deliberate induction of an immune response is known as **immunization**. Experimental immunizations are routinely carried out by injecting the test antigen into the animal or human subject. The route, dose, and form in which antigen is administered can profoundly affect whether a response occurs and the type of response that is produced, and are considered in Sections A-1–A-4. The induction of protective immune responses against common microbial pathogens in humans is often called vaccination, although this term is correctly only applied to the induction of immune responses against smallpox by immunizing with the cross-reactive cowpox virus, vaccinia.

To determine whether an immune response has occurred and to follow its course, the immunized individual is monitored for the appearance of immune reactants directed at the specific antigen. Immune responses to most antigens elicit the production of both specific antibodies and specific effector T cells. Monitoring the antibody response usually involves the analysis of relatively crude preparations of **antiserum** (plural: **antisera**). The **serum** is the fluid phase of clotted blood, which, if taken from an immunized individual, is called antiserum because it contains specific antibodies against the immunizing antigen as well as other soluble serum proteins. To study immune responses mediated by T cells, blood lymphocytes or cells from lymphoid organs such as the spleen are tested; T-cell responses are more commonly studied in experimental animals than in humans.

Any substance that can elicit an immune response is said to be **immunogenic** and is called an **immunogen**. There is a clear operational distinction between an immunogen and an antigen. An antigen is defined as any substance that can bind to a specific antibody. All antigens therefore have the potential to elicit specific antibodies, but some need to be attached to an immunogen in order to do so. This means that although all immunogens are antigens, not all antigens are immunogenic. The antigens used most frequently in experimental immunology are proteins, and antibodies against proteins are of enormous utility in experimental biology and medicine. Purified proteins are, however, not always highly immunogenic, and to provoke an immune response they have to be administered with an adjuvant (see Section A-4). Carbohydrates, nucleic acids, and other types of molecules are all potential antigens, but will often only induce an immune response if attached to a protein carrier. Thus, the immunogenicity of protein antigens determines the outcome of virtually every immune response.

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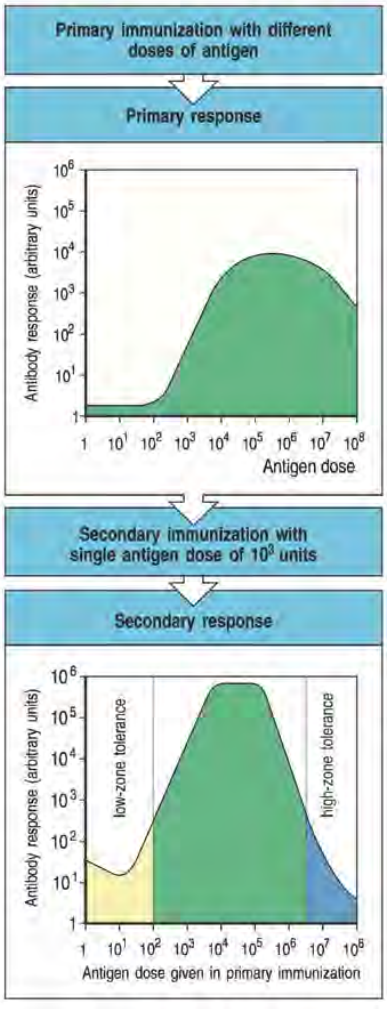


Fig. A.1 The dose of antigen used in an initial immunization affects the primary and secondary antibody response. The typical antigen dose–response curve shown here illustrates the influence of dose on both a primary antibody response (amounts of antibody produced expressed in arbitrary units) and the effect of the dose used for priming on a secondary antibody response elicited by a dose of antigen of 10^3 arbitrary mass units. Very low doses of antigen do not cause an immune response at all. Slightly higher doses seem to inhibit specific antibody production, an effect known as low-zone tolerance. Above these doses there is a steady increase in the response with antigen dose to reach a broad optimum. Very high doses of antigen also inhibit immune responsiveness to a subsequent challenge, a phenomenon known as high-zone tolerance.

Antisera generated by immunization with even the simplest antigen will contain many different antibody molecules that bind to the immunogen in slightly different ways. Some of the antibodies in an antiserum are cross-reactive. A **cross-reaction** is defined as the binding of an antibody to an antigen other than the immunogen; most antibodies cross-react with closely related antigens but, on occasion, some bind antigens with no clear relationship to the immunogen. These cross-reacting antibodies can create problems when the antiserum is used to detect a specific antigen. They can be removed from an antiserum by **absorption** with the cross-reactive antigen, leaving behind the antibodies that bind only to the immunogen. Absorption can be performed by affinity chromatography using immobilized antigen, a technique that is also used for the purification of antibodies or antigens (see Section A-5). Most problems of cross-reactivity can be avoided, however, by making monoclonal antibodies (see Section A-12).

Although almost any structure can be recognized by antibody as an antigen, usually only proteins elicit fully developed adaptive immune responses. This is because proteins have the ability to engage T cells, which contribute to inducing most antibody responses and are required for immunological memory. Proteins engage T cells because the T cells recognize antigens as peptide fragments of proteins bound to major histocompatibility complex (MHC) molecules. An adaptive immune response that includes immunological memory can be induced by nonpeptide antigens only when they are attached to a protein carrier that can engage the necessary T cells (see Section 10-3 and Fig. 10.5).

Immunological memory is produced as a result of the initial or **primary immunization**, which evokes the **primary immune response**. This is also known as **priming**, because the animal or person is now 'primed' like a pump to mount a more potent response to subsequent challenges with the same antigen. The response to each immunization is increasingly intense, so that **secondary**, **tertiary**, and subsequent responses are of increasing magnitude (Fig. A.1). Repetitive challenge with antigen to achieve a heightened state of immunity is known as **hyperimmunization**.

Certain properties of a protein that favor the priming of an adaptive immune response have been defined by studying antibody responses to simple natural proteins such as hen egg-white lysozyme and to synthetic polypeptide antigens (Fig. A.2). The larger and more complex a protein, and the more distant its relationship to self proteins, the more likely it is to elicit a response. This is because such responses depend on the proteins being degraded into peptides that can bind to MHC molecules, and on the subsequent recognition of these peptide:MHC complexes by T cells (see Chapter 6). The larger and more distinct the protein antigen, the more likely it is to contain such peptides. Particulate or aggregated antigens are more immunogenic because they are taken up more efficiently by the specialized antigen-presenting cells responsible for initiating a response. Indeed, small soluble proteins are unable to induce a response unless they are made to aggregate in some way. Many vaccines, for example, use aggregated protein antigens to potentiate the immune response.

A-1 Haptens.

Small organic molecules of simple structure, such as phenyl arsonates and nitrophenyls, do not provoke antibodies when injected by themselves. However, antibodies can be raised against them if the molecule is attached covalently, by simple chemical reactions, to a protein carrier. Such small molecules were termed **haptens** (from the Greek *haptein*, to fasten) by the immunologist Karl Landsteiner, who first studied them in the early 20th

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Factors that influence the immunogenicity of proteins		
Parameter	**Increased immunogenicity**	**Decreased immunogenicity**
Size	Large	Small (MW<2500)
Dose	Intermediate	High or low
Route	Subcutaneous > intraperitoneal > intravenous or intragastric	
Composition	Complex	Simple
Form	Particulate	Soluble
	Denatured	Native
Similarity to self protein	Multiple differences	Few differences
Adjuvants	Slow release	Rapid release
	Bacteria	No bacteria
Interaction with host MHC	Effective	Ineffective

Fig. A.2 Intrinsic properties and extrinsic factors that affect the immunogenicity of proteins. MW, molecular weight in daltons.

century. He found that animals immunized with a hapten–carrier conjugate produced three distinct sets of antibodies (Fig. A.3). One set comprised hapten-specific antibodies that reacted with the same hapten on any carrier, as well as with free hapten. The second set of antibodies was specific for the carrier protein, as shown by their ability to bind both the hapten-modified and unmodified carrier protein. Finally, some antibodies reacted only with the specific conjugate of hapten and carrier used for immunization. Landsteiner studied mainly the antibody response to the hapten, as these small molecules could be synthesized in many closely related forms. He observed that antibodies raised against a particular hapten bind that hapten but, in general, fail to bind even very closely related chemical structures. The binding of haptens by anti-hapten antibodies has played an important part in defining the precision of antigen binding by antibody molecules. Anti-hapten antibodies are also important medically because they mediate allergic reactions to penicillin and other compounds that elicit antibody responses when they attach to self proteins (see Section 14-10).

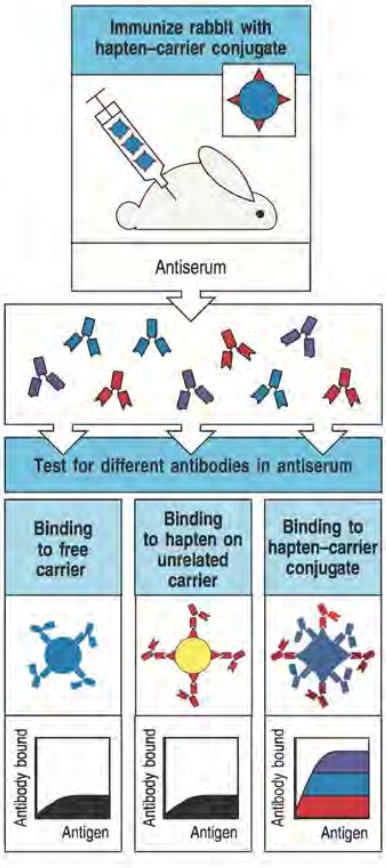


Fig. A.3 Antibodies can be elicited by small chemical groups called haptens only when the hapten is linked to an immunogenic protein carrier. Three types of antibodies are produced. One set (blue) binds the carrier protein alone and is called carrier-specific. One set (red) binds to the hapten on any carrier or to free hapten in solution and is called hapten-specific. One set (purple) only binds the specific conjugate of hapten and carrier used for immunization, apparently binding to sites at which the hapten joins the carrier, and is called conjugate-specific. The amount of antibody of each type in this serum is shown schematically in the graphs at the bottom; note that the original antigen binds more antibody than the sum of anti-hapten and anti-carrier antibodies as a result of the additional binding of conjugate-specific antibody.

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A-2 Routes of immunization.

The route by which antigen is administered affects both the magnitude and the type of response obtained. The most common routes by which antigen is introduced experimentally or as a vaccine into the body are injection into tissue by **subcutaneous** (**s.c.**) injection into the fatty layer just below the dermis, or by **intradermal** (**i.d.**) or **intramuscular** (**i.m.**) injection; by **intravenous** (**i.v.**) injection or transfusion directly into the bloodstream; into the gastrointestinal tract by oral administration; and into the respiratory tract by **intranasal** (**i.n.**) administration or inhalation.

Antigens injected subcutaneously generally elicit the strongest responses, most probably because the antigen is taken up by Langerhans cells in the skin and efficiently presented in local lymph nodes, and so this is the method most commonly used when the object of the experiment is to elicit specific antibodies or T cells against a given antigen. Antigens injected or transfused directly into the bloodstream tend to induce immune unresponsiveness or tolerance unless they bind to host cells or are in the form of aggregates that are readily taken up by antigen-presenting cells.

Antigen administration via the gastrointestinal tract is used mostly in the study of allergy. It has distinctive effects, frequently eliciting a local antibody response in the intestinal lamina propria, while producing a systemic state of tolerance that manifests as a diminished response to the same antigen if subsequently administered in immunogenic form elsewhere in the body (see Chapter 12). This 'split tolerance' may be important in avoiding allergy to antigens in food, because the local response prevents food antigens from entering the body, while the inhibition of systemic immunity helps to prevent the formation of IgE antibodies, which are the cause of such allergies (see Chapter 14).

Introduction of antigen into the respiratory tract is also used mainly in the study of allergy. Protein antigens that enter the body through the respiratory epithelium tend to elicit allergic responses, for reasons that are not clear.

A-3 Effects of antigen dose.

The magnitude of the immune response depends on the dose of immunogen administered. Below a certain threshold dose, most proteins do not elicit any immune response. Above the threshold dose, there is a gradual increase in the response as the dose of antigen is increased, until a broad plateau level is reached, followed by a decline at very high antigen doses (see Fig. A.1). Because most infectious agents enter the body in small numbers, immune responses are generally elicited only by pathogens that multiply to a level sufficient to exceed the antigen dose threshold. The broad response optimum allows the system to respond to infectious agents across a wide range of doses. At very high antigen doses, the immune response is inhibited, which may be important in maintaining tolerance to abundant self proteins such as plasma proteins. In general, secondary and subsequent immune responses occur at lower antigen doses and achieve higher plateau values, which is a sign of immunological memory. However, under some conditions, very low or very high doses of antigen may induce specific unresponsive states, known respectively as acquired low-zone or high-zone tolerance.

A-4 Adjuvants.

Most proteins are poorly immunogenic or nonimmunogenic when administered by themselves. Strong adaptive immune responses to protein antigens almost always require that the antigen be injected in a mixture known as an

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adjuvant. An adjuvant is any substance that enhances the immunogenicity of substances mixed with it. Adjuvants differ from protein carriers in that they do not form stable linkages with the immunogen. Furthermore, adjuvants are needed primarily for initial immunizations, whereas carriers are required to elicit not only primary but also subsequent responses to haptens. Commonly used adjuvants are listed in Fig. A.4.

Adjuvants can enhance immunogenicity in two different ways. First, adjuvants convert soluble protein antigens into particulate material, which is more readily ingested by antigen-presenting cells such as macrophages. For example, the antigen can be adsorbed on particles of the adjuvant (such as alum), made particulate by emulsification in mineral oils, or incorporated into the colloidal particles of immune stimulatory complexes (ISCOMs). This enhances immunogenicity somewhat, but such adjuvants are relatively weak unless they also contain bacteria or bacterial products. Such microbial constituents are the second means by which adjuvants enhance immunogenicity, and although their exact contribution to enhancing immunogenicity is unknown, they are clearly the more important component of an adjuvant. Microbial products may signal macrophages or dendritic cells to become more effective antigen-presenting cells (see Section 3-10). One of their effects is to induce the production of inflammatory cytokines and potent local inflammatory responses; this effect is probably intrinsic to their activity in enhancing responses, but precludes their use in humans.

Nevertheless, some human vaccines contain microbial antigens that can also act as effective adjuvants. For example, purified constituents of the bacterium *Bordetella pertussis*, which is the causative agent of whooping cough, are used as both antigen and adjuvant in the triplex DPT (diphtheria, pertussis, tetanus) vaccine against these diseases.

B cells contribute to adaptive immunity by secreting antibodies, and the response of B cells to an injected immunogen is usually measured by

Adjuvants that enhance immune responses

Adjuvant name	Composition	Mechanism of action
Incomplete Freund's adjuvant	Oil-in-water emulsion	Delayed release of antigen; enhanced uptake by macrophages
Complete Freund's adjuvant	Oil-in-water emulsion with dead mycobacteria	Delayed release of antigen; enhanced uptake by macrophages; induction of co-stimulators in macrophages
Freund's adjuvant with MDP	Oil-in-water emulsion with muramyldipeptide (MDP), a constituent of mycobacteria	Similar to complete Freund's adjuvant
Alum (aluminum hydroxide)	Aluminum hydroxide gel	Delayed release of antigen; enhanced macrophage uptake
Alum plus *Bordetella pertussis*	Aluminum hydroxide gel with killed *B. pertussis*	Delayed release of antigen; enhanced uptake by macrophages; induction of co-stimulators
Immune stimulatory complexes (ISCOMs)	Matrix of Quil A containing viral proteins	Delivers antigen to cytosol; allows induction of cytotoxic T cells

Fig. A.4 Common adjuvants and their use. Adjuvants are mixed with the antigen and usually render it particulate, which helps to retain the antigen in the body and promotes uptake by macrophages. Most adjuvants include bacteria or bacterial components that stimulate macrophages, aiding in the induction of the immune response. ISCOMs are small micelles of the detergent Quil A; when viral proteins are placed in these micelles, they apparently fuse with the antigen-presenting cell, allowing the antigen to enter the cytosol. Thus, the antigen-presenting cell can stimulate a response to the viral protein, much as a virus infecting these cells would stimulate an antiviral response.

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analyzing the specific antibody produced in a **humoral immune response**. This is most conveniently achieved by assaying the antibody that accumulates in the fluid phase of the blood or **plasma**; such antibodies are known as circulating antibodies. Circulating antibody is usually measured by collecting blood, allowing it to clot, and then isolating the serum from the clotted blood. The amount and characteristics of the antibody in the resulting antiserum are then determined using the assays we describe in Sections A-5–A-11.

The most important characteristics of an antibody response are the specificity, amount, isotype or class, and affinity of the antibodies produced. The **specificity** determines the ability of the antibody to distinguish the immunogen from other antigens. The amount of antibody can be determined in many different ways and is a function of the number of responding B cells, their rate of antibody synthesis, and the persistence of the antibody after production. The persistence of an antibody in the plasma and extracellular fluid bathing the tissues is determined mainly by its isotype or class (see Sections 5-12 and 10-14); each isotype has a different half-life *in vivo*. The isotypic composition of an antibody response also determines the biological functions these antibodies can perform and the sites in which antibody will be found. Finally, the strength of binding of the antibody to its antigen in terms of a single antigen-binding site binding to a monovalent antigen is termed its **affinity**; the total binding strength of a molecule with more than one binding site is called its **avidity**. Binding strength is important, because the higher the affinity of the antibody for its antigen, the less antibody is required to eliminate the antigen, because antibodies with higher affinity will bind at lower antigen concentrations. All these parameters of the humoral immune response help to determine the capacity of that response to protect the host from infection.

Antibody molecules are highly specific for their corresponding antigen, being able to detect one molecule of a protein antigen out of more than 10^8 similar molecules. This makes antibodies both easy to isolate and easy to study, and invaluable as probes of biological processes. Whereas standard chemistry would have great difficulty in distinguishing between two such closely related proteins as human and pig insulin, or two such closely related structures as *ortho*- and *para*-nitrophenyl, antibodies can be made that discriminate between these two structures absolutely. The value of antibodies as molecular probes has stimulated the development of many sensitive and highly specific techniques to measure their presence, to determine their specificity and affinity for a range of antigens, and to ascertain their functional capabilities. Many standard techniques used throughout biology exploit the specificity and stability of antigen binding by antibodies. Comprehensive guides to the conduct of these antibody assays are available in many books on immunological methodology; we will illustrate here only the most important techniques, especially those used in studying the immune response itself.

Some assays for antibody measure the direct binding of the antibody to its antigen. Such assays are based on **primary interactions**. Others determine the amount of antibody present by the changes it induces in the physical state of the antigen, such as the precipitation of soluble antigen or the clumping of antigenic particles; these are called **secondary interactions**. Both types of assay can be used to measure the amount and specificity of the antibodies produced after immunization, and both can be applied to a wide range of other biological questions.

Because assays for antibody were originally conducted using antisera from immune individuals, they are commonly referred to as **serological assays**, and the use of antibodies is often called **serology**. The amount of antibody is usually determined by antigen-binding assays after titration of the antiserum by serial dilution, and the point at which binding falls to 50% of the maximum is usually referred to as the **titer** of an antiserum.

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The detection, measurement, and characterization of antibodies, and their use as research and diagnostic tools.

A-5 Affinity chromatography.

Specific antibody can be isolated from an antiserum by **affinity chromatography**, which exploits the specific binding of antibody to antigen held on a solid matrix (Fig. A.5). Antigen is bound covalently to small, chemically reactive beads, which are loaded into a column, and the antiserum is allowed to pass over the beads. The specific antibodies bind, while all the other proteins in the serum, including antibodies against other substances, can be washed away. The specific antibodies are then eluted, typically by lowering the pH to 2.5 or raising it to greater than 11. Antibodies bind stably under physiological conditions of salt concentration, temperature, and pH, but the binding is reversible because the bonds are noncovalent. Affinity chromatography can also be used to purify antigens from complex mixtures by using beads coated with specific antibody. The technique is known as affinity chromatography because it separates molecules on the basis of their affinity for one another.

A-6 Radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and competitive inhibition assay.

Radioimmunoassay (**RIA**) and **enzyme-linked immunosorbent assay** (**ELISA**) are direct binding assays for antibody (or antigen); both work on the same principle, but the means of detecting specific binding is different. Radioimmunoassays are commonly used to measure the levels of hormones in blood and tissue fluids, while ELISA assays are frequently used in viral diagnostics, for example in detecting cases of infection with the human immunodeficiency virus (HIV), which is the cause of AIDS. For both these methods one needs a pure preparation of a known antigen or antibody, or both, in order to standardize the assay. We will describe the assay with a sample of pure antibody, which is the more usual case, but the principle is similar if pure antigen is used instead. In RIA for an antigen, pure antibody against that antigen is radioactively labeled, usually with ^{125}I; for ELISA, an enzyme is linked chemically to the antibody. The unlabeled component, which in this case would be

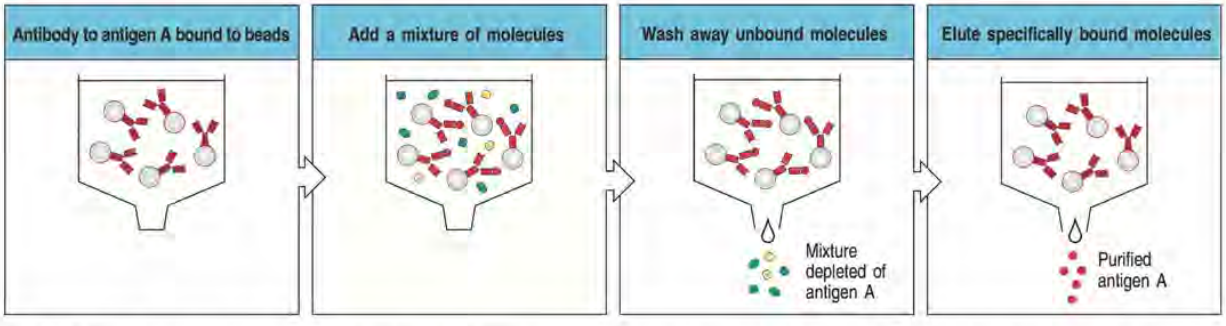


Fig. A.5 Affinity chromatography uses antigen–antibody binding to purify antigens or antibodies. To purify a specific antigen from a complex mixture of molecules, a monoclonal antibody is attached to an insoluble matrix, such as chromatography beads, and the mixture of molecules is passed over the matrix. The specific antibody binds the antigen of interest; other molecules are washed away. Specific antigen is then eluted by altering the pH, which can usually disrupt antibody–antigen bonds. Antibodies can be purified in the same way on beads coupled to antigen (not shown).

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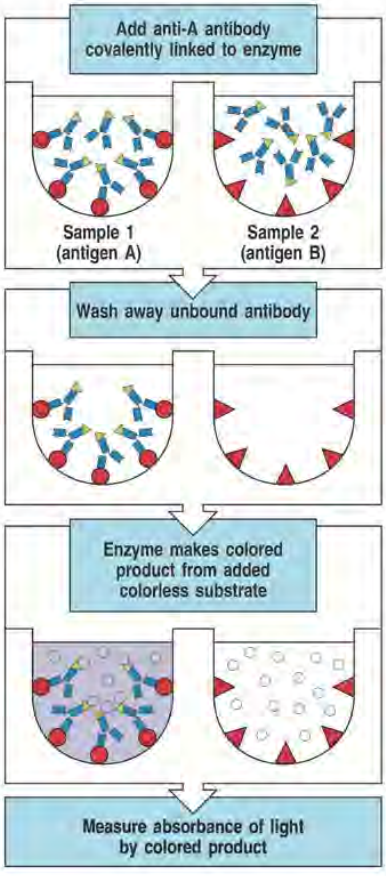


Fig. A.6 The principle of the enzyme-linked immunosorbent assay (ELISA). To detect antigen A, purified antibody specific for antigen A is linked chemically to an enzyme. The samples to be tested are coated onto the surface of plastic wells to which they bind nonspecifically; residual sticky sites on the plastic are blocked by adding irrelevant proteins (not shown). The labeled antibody is then added to the wells under conditions where nonspecific binding is prevented, so that only binding to antigen A causes the labeled antibody to be retained on the surface. Unbound labeled antibody is removed from all wells by washing, and bound antibody is detected by an enzyme-dependent color-change reaction. This assay allows arrays of wells known as microtiter plates to be read in fiberoptic multichannel spectrometers, greatly speeding the assay. Modifications of this basic assay allow antibody or antigen in unknown samples to be measured as shown in Figs A.7 and A.30 (see also Section A-10).

antigen, is attached to a solid support, such as the wells of a plastic multiwell plate, which will adsorb a certain amount of any protein.

The labeled antibody is allowed to bind to the unlabeled antigen, under conditions in which nonspecific adsorption is blocked, and any unbound antibody and other proteins are washed away. Antibody binding in RIA is measured directly in terms of the amount of radioactivity retained by the coated wells, whereas in ELISA the binding is detected by a reaction that converts a colorless substrate into a colored reaction product (Fig. A.6). The color change can be read directly in the reaction tray, making data collection very easy, and ELISA also avoids the hazards of radioactivity. This makes ELISA the preferred method for most direct-binding assays. Labeled anti-immunoglobulin antibodies (see Section A-10) can also be used in RIA or ELISA to detect the binding of unlabeled antibody to unlabeled antigen-coated plates. In this case, the labeled anti-immunoglobulin antibody is used in what is termed a 'second layer.' The use of such a second layer also amplifies the signal, because at least two molecules of the labeled anti-immunoglobulin antibody are able to bind to each unlabeled antibody. RIA and ELISA can also be carried out with unlabeled antibody stuck to the plates and labeled antigen added.

A modification of ELISA known as a **capture** or **sandwich ELISA** (or more generally as an **antigen-capture assay**) can be used to detect secreted products such as cytokines. Rather than the antigen being directly attached to a plastic plate, antigen-specific antibodies are bound to the plate. These are able to bind antigen with high affinity, and thus concentrate it on the surface of the plate, even with antigens that are present in very low concentrations in the initial mixture. A separate labeled antibody that recognizes a different epitope from that recognized by the immobilized first antibody is then used to detect the bound antigen.

These assays illustrate two crucial aspects of all serological assays. First, at least one of the reagents must be available in a pure, detectable form in order to obtain quantitative information. Second, there must be a means of separating the bound fraction of the labeled reagent from the unbound, free fraction so that the percentage of specific binding can be determined. Normally, this separation is achieved by having the unlabeled partner trapped on a solid support. Labeled molecules that do not bind can then be washed away, leaving just the labeled partner that has bound. In Fig. A.6, the unlabeled antigen is attached to the well and the labeled antibody is trapped by binding to it. The separation of bound from free is an essential step in every assay that uses antibodies.

RIA and ELISA do not allow one to measure directly the amount of antigen or antibody in a sample of unknown composition, because both depend on the binding of a pure labeled antigen or antibody. There are various ways around this problem, one of which is to use a **competitive inhibition assay**, as shown in Fig. A.7. In this type of assay, the presence and amount of a particular antigen in an unknown sample are determined by its ability to compete with a labeled reference antigen for binding to an antibody attached to a plastic well. A standard curve is first constructed by adding various amounts of a known, unlabeled standard preparation; the assay can then measure the amount of antigen in unknown samples by comparison with the standard. The competitive binding assay can also be used for measuring antibody in a sample of unknown composition by attaching the appropriate antigen to the plate and measuring the ability of the test sample to inhibit the binding of a labeled specific antibody.

A-7 Hemagglutination and blood typing.

The direct measurement of antibody binding to antigen is used in most quantitative serological assays. However, some important assays are based on the

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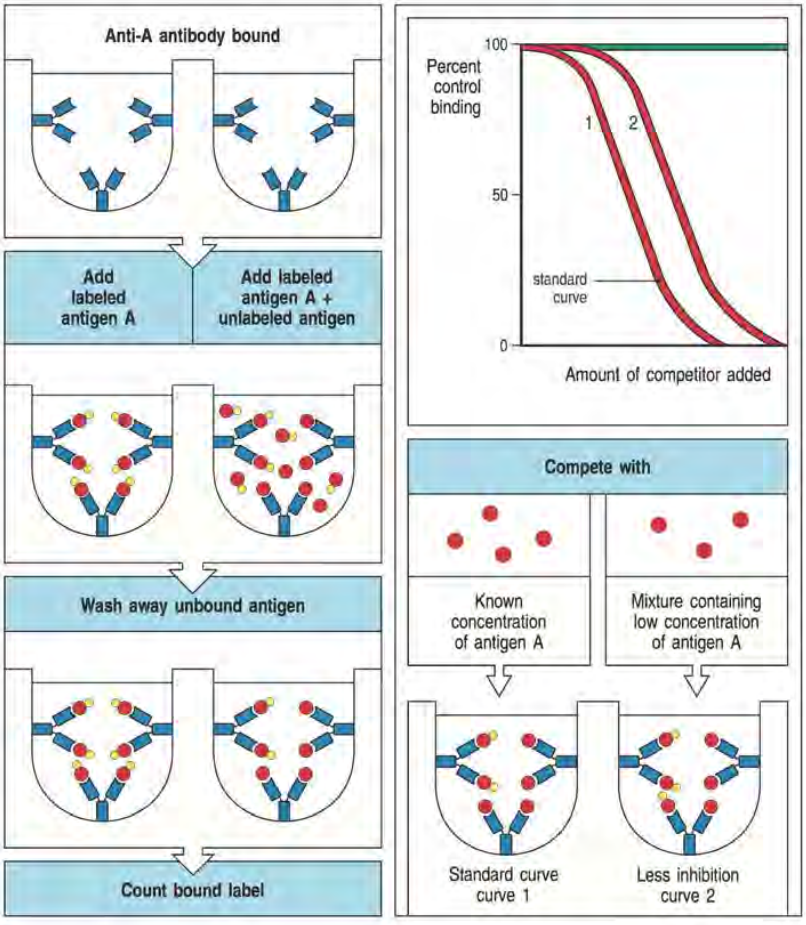


Fig. A.7 Competitive inhibition assay for antigen in unknown samples. A fixed amount of unlabeled antibody is attached to a set of wells, and a standard reference preparation of a labeled antigen is bound to it. Unlabeled standard or test samples are then added in various amounts and the displacement of labeled antigen is measured, generating characteristic inhibition curves. A standard curve is obtained by using known amounts of unlabeled antigen identical to that used as the labeled species, and comparison with this curve allows the amount of antigen in unknown samples to be calculated. The green line on the graph represents a sample lacking any substance that reacts with anti-A antibodies.

ability of antibody binding to alter the physical state of the antigen it binds to. These secondary interactions can be detected in a variety of ways. For instance, when the antigen is displayed on the surface of a large particle such as a bacterium, antibodies can cause the bacteria to clump or **agglutinate**. The same principle applies to the reactions used in blood typing, only here the target antigens are on the surface of red blood cells and the clumping reaction caused by antibodies against them is called **hemagglutination** (from the Greek *haima*, blood).

Hemagglutination is used to determine the **ABO blood group** of blood donors and transfusion recipients. Clumping or agglutination is induced by antibodies or agglutinins called anti-A or anti-B that bind to the A or B blood group substances, respectively (Fig. A.8). These blood group antigens are arrayed in many copies on the surface of the red blood cell, causing the cells to agglutinate when cross-linked by antibodies. Because hemagglutination involves the cross-linking of blood cells by the simultaneous binding of antibody molecules to identical antigens on different cells, this reaction also demonstrated that each antibody molecule must have at least two identical antigen-binding sites.

A-8 Precipitin reaction.

When sufficient quantities of antibody are mixed with soluble macromolecular antigens, a visible precipitate consisting of large aggregates of antigen cross-linked by antibody molecules can form. The amount of precipitate

Fig. A.8 Hemagglutination is used to type blood groups and match compatible donors and recipients for blood transfusion. Common gut bacteria bear antigens that are similar or identical to blood group antigens, and these stimulate the formation of antibodies against these antigens in individuals who do not bear the corresponding antigen on their own red blood cells (left column); thus, type O individuals, who lack A and B, have both anti-A and anti-B antibodies, whereas type AB individuals have neither. The pattern of agglutination of the red blood cells of a transfusion donor or recipient with anti-A and anti-B antibodies reveals the individual's ABO blood group. Before transfusion, the serum of the recipient is also tested for antibodies that agglutinate the red blood cells of the donor, and vice versa, a procedure called a cross-match, which may detect potentially harmful antibodies against other blood groups that are not part of the ABO system.

Serum from individuals of type	Red blood cells from individuals of type: O Express the carbohydrate structures: R–GlcNAc–Gal (Fuc)	Red blood cells from individuals of type: A Express the carbohydrate structures: R–GlcNAc–Gal–GalNAc (Fuc)	Red blood cells from individuals of type: B Express the carbohydrate structures: R–GlcNAc–Gal–Gal (Fuc)	Red blood cells from individuals of type: AB Express the carbohydrate structures: R–GlcNAc–Gal–GalNAc (Fuc) + R–GlcNAc–Gal–Gal (Fuc)
O Anti-A and anti-B antibodies	no agglutination	agglutination	agglutination	agglutination
A Anti-B antibodies	no agglutination	no agglutination	agglutination	agglutination
B Anti-A antibodies	no agglutination	agglutination	no agglutination	agglutination
AB No antibodies to A or B	no agglutination	no agglutination	no agglutination	no agglutination

depends on the amounts of antigen and antibody, and on the ratio between them (Fig. A.9). This **precipitin reaction** provided the first quantitative assay for antibody, but is now seldom used in immunology. However, it is important to understand the interaction of antigen with antibody that leads to this reaction, because the production of **antigen:antibody complexes**, also known as **immune complexes**, *in vivo* occurs in almost all immune responses and can occasionally cause significant pathology (see Chapters 14 and 15).

In the precipitin reaction, various amounts of soluble antigen are added to a fixed amount of serum containing antibody. As the amount of antigen added increases, the amount of precipitate generated also increases up to a maximum and then declines (see Fig. A.9). When small amounts of antigen are added, antigen:antibody complexes are formed under conditions of antibody excess so that each molecule of antigen is bound by antibody and cross-linked to other molecules of antigen. When large amounts of antigen are added, only small antigen:antibody complexes can form, and these are often soluble in this zone of antigen excess. Between these two zones, all of the antigen and antibody is found in the precipitate, generating a zone of equivalence. At equivalence, very large lattices of antigen and antibody are formed by cross-linking. Although all antigen:antibody complexes can potentially produce disease, the small, soluble immune complexes formed in the zone of antigen excess may persist and cause pathology *in vivo*.

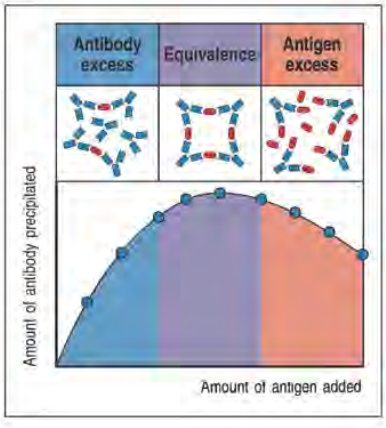


Fig. A.9 Antibody can precipitate soluble antigen. Analysis of the precipitate can generate a precipitin curve. Different amounts of antigen are added to a fixed amount of antibody, and precipitates form by antibody cross-linking of antigen molecules. The precipitate is recovered and the amount of precipitated antibody is measured; the supernatant is tested for residual antigen or antibody. This defines zones of antibody excess, equivalence, and antigen excess. At equivalence, the largest antigen:antibody complexes form. In the zone of antigen excess, some of the immune complexes are too small to precipitate. These soluble immune complexes can cause pathological damage to small blood vessels when they form *in vivo* (see Chapter 15).

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The precipitin reaction is affected by the number of binding sites that each antibody has for antigen, and by the maximum number of antibodies that can be bound by an antigen molecule or particle at any one time. These quantities are defined as the **valence** of the antibody and the valence of the antigen: the valence of both the antibodies and the antigen must be two or greater before any precipitation can occur. The valence of an antibody depends on its structural class (see Section 5-12).

Antigen will be precipitated only if it has several antibody-binding sites. This condition is usually satisfied in macromolecular antigens, which have a complex surface with binding sites for several different antibodies. The site on an antigen to which each distinct antibody molecule binds is called an **antigenic determinant** or an **epitope**. Steric considerations limit the number of distinct antibody molecules that can bind to a single antigen molecule at any one time, however, because antibody molecules binding to epitopes that partly overlap will compete for binding. For this reason, the valence of an antigen is almost always less than the number of epitopes on the antigen (Fig. A.10).

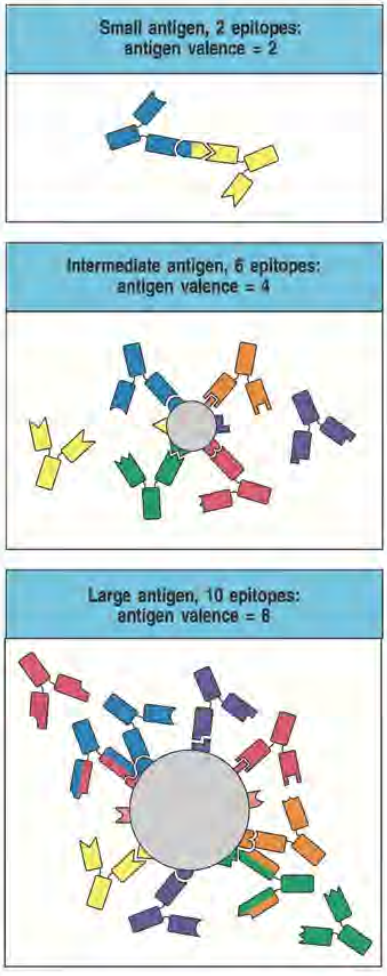


Fig. A.10 Different antibodies bind to distinct epitopes on an antigen molecule. The surface of an antigen possesses many potential antigenic determinants or epitopes, distinct sites to which an antibody can bind. The number of antibody molecules that can bind to a molecule of antigen at one time defines the antigen's valence. Steric considerations can limit the number of different antibodies that bind to the surface of an antigen at any one time (middle and bottom panels), so that the number of epitopes on an antigen is always greater than or equal to its valence.

A-9 Equilibrium dialysis: measurement of antibody affinity and avidity.

The affinity of an antibody is the strength of binding of a monovalent ligand to a single antigen-binding site on the antibody. The affinity of an antibody that binds small antigens, such as haptens, that can diffuse freely across a dialysis membrane can be determined directly by the technique of **equilibrium dialysis**. A known amount of antibody, whose molecules are too large to cross a dialysis membrane, is placed in a dialysis bag and offered various amounts of antigen. Molecules of antigen that bind to the antibody are no longer free to diffuse across the dialysis membrane, so only the unbound molecules of antigen equilibrate across it. By measuring the concentration of antigen inside the bag and in the surrounding fluid, one can determine the amount of the antigen that is bound as well as the amount that is free when equilibrium has been achieved. Given that the amount of antibody present is known, the affinity of the antibody and the number of specific binding sites for the antigen per molecule of antibody can be determined from this information. The data are usually analyzed using a **Scatchard analysis** (Fig. A.11); such analyses were used to demonstrate that a molecule of IgG antibody has two identical antigen-binding sites.

Whereas affinity measures the strength of binding of an antigenic determinant to a single antigen-binding site, an antibody reacting with an antigen that has multiple identical epitopes or with the surface of a pathogen will often bind the same molecule or particle with both of its antigen-binding sites. This increases the apparent strength of binding, because both binding sites must release at the same time for the two molecules to dissociate. This is often referred to as **cooperativity** in binding, but it should not be confused with the cooperative binding found in a protein such as hemoglobin, in which binding of ligand at one site enhances the affinity of a second binding site for its ligand. The overall strength of binding of an antibody molecule to an antigen or particle is called its avidity (Fig. A.12). For IgG antibodies, bivalent binding can significantly increase avidity; in IgM antibodies, which have 10 identical antigen-binding sites, the affinity of each site for a monovalent antigen is usually quite low, but the avidity of binding of the whole antibody to a surface such as a bacterium that displays multiple identical epitopes can be very high.

A-10 Anti-immunoglobulin antibodies.

A general approach to the detection of bound antibody that avoids the need to label each preparation of antibody molecules is to detect bound, unlabeled

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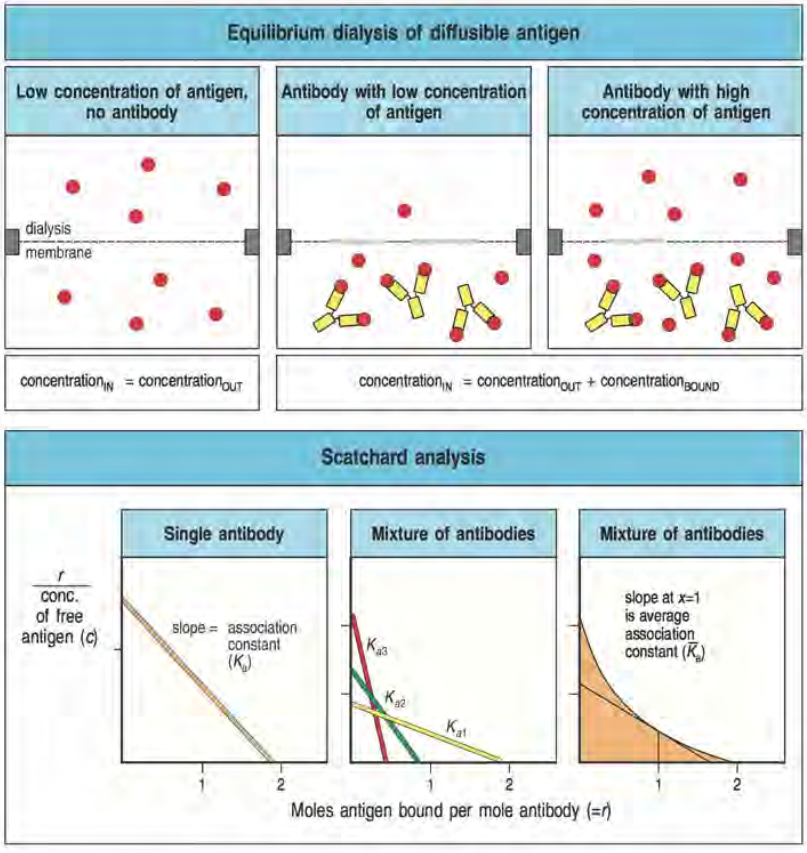


Fig. A.11 The affinity and valence of an antibody can be determined by equilibrium dialysis. A known amount of antibody is placed in the bottom half of a dialysis chamber and exposed to different amounts of a diffusible monovalent antigen, such as a hapten. At equilibrium, the concentration of free antigen will be the same on each side of the membrane, so that at each concentration of antigen added, the fraction of the antigen bound is determined from the difference in concentration of total antigen in the top and bottom chambers. This information can be transformed into a Scatchard plot as shown here. In Scatchard analysis, the ratio r/c (where r = moles of antigen bound per mole of antibody and c = molar concentration of free antigen) is plotted against r. The number of binding sites per antibody molecule can be determined from the value of r at infinite free-antigen concentration, where r/free = 0, in other words at the x-axis intercept. The analysis of a monoclonal IgG antibody molecule, in which there are two identical antigen-binding sites per molecule, is shown in the left panel. The slope of the line is determined by the affinity of the antibody molecule for its antigen; if all the antibody molecules in a preparation are identical, as for this monoclonal antibody, then a straight line is obtained whose slope is equal to $-K_a$, where K_a is the association (or affinity) constant, and the dissociation constant $K_d = 1/K_a$. However, antiserum raised even against a simple antigenic determinant such as a hapten contains a heterogeneous population of antibody molecules (see Section A-1). Each antibody molecule would, if isolated, make up part of the total and give a straight line whose x-axis intercept is less than two, because this antibody molecule contains only a fraction of the total binding sites in the population (middle panel). As a mixture, they give curved lines with an x-axis intercept of two for which an average affinity ($\overline{K_a}$) can be determined from the slope of this line at a concentration of antigen where 50% of the sites are bound, or at x = 1 (right panel). The association constant determines the equilibrium state of the reaction Ag + Ab = Ag:Ab, where Ag = antigen and Ab = antibody, and K_a = [Ag:Ab]/[Ag][Ab]. This constant reflects the 'on' and 'off' rates for antigen binding to the antibody; with small antigens such as haptens, binding is usually as rapid as diffusion allows, whereas differences in 'off' rates determine the affinity constant. However, with larger antigens the 'on' rate may also vary as the interaction becomes more complex.

antibody with a labeled antibody specific for immunoglobulins themselves. Immunoglobulins, like other proteins, are immunogenic when used to immunize individuals of another species. The majority of **anti-immunoglobulin antibodies** raised in this way recognize conserved features shared by all immunoglobulin molecules of the immunizing species. Others can be specific for immunoglobulin chains, such as the heavy or light chains, or for individual isotypes. Antibodies raised by immunization of goats with mouse IgG are commonly used in experimental immunology. Such goat anti-mouse IgG antibodies can be purified using affinity chromatography, then labeled and used as a general probe for bound IgG antibodies. Anti-immunoglobulin antisera have found many uses in clinical medicine and biological research since their introduction. Fluorescently labeled anti-immunoglobulin antibodies are now widely used both in immunology and other areas of biology as secondary reagents for detecting specific antibodies bound, for example, to cell structures (see Sections A-14 and A-16). Labeled anti-immunoglobulin antibodies can also be used in RIA or ELISA (see Section A-6) to detect the binding of unlabeled antibody to antigen-coated plates.

When an immunoglobulin is used as an antigen to immunize a different species of animal, it will be treated like any other foreign protein and will elicit an antibody response. Anti-immunoglobulin antibodies can be made that recognize the amino acids that characterize the isotype of the injected antibody. Such **anti-isotypic antibodies** recognize all immunoglobulins of the same isotype in all members of the species from which the injected antibody came.

It is also possible to raise antibodies that recognize differences in immunoglobulins from members of the same species that are due to the presence of multiple alleles of the individual C genes in the population (genetic

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polymorphism). Such allelic variants are called **allotypes**. In contrast to anti-isotypic antibodies, anti-allotypic antibodies will recognize immunoglobulin of a particular isotype in only some members of a species. Finally, because individual antibodies differ in their variable regions, one can raise antibodies against unique features of the antigen-binding site, which are called **idiotypes**.

A schematic picture of the differences between idiotypes, allotypes, and isotypes is given in Fig. A.13. Historically, the main features of immunoglobulins were defined by using isotypic and allotypic genetic markers identified by antisera raised in different species or in genetically distinct members of the same species. The independent segregation of allotypic and isotypic markers revealed the existence of separate heavy-chain, κ, and λ genes. Such anti-idiotypic, allotypic, and isotypic antibodies are still enormously useful in detecting antibodies and B cells in scientific experimentation and medical diagnostics.

Antibodies specific for individual immunoglobulin isotypes can be produced by immunizing an animal of a different species with a pure preparation of one isotype and then removing those antibodies that cross-react with immunoglobulins of other isotypes by using affinity chromatography (see Section A-5). Anti-isotype antibodies can be used to measure how much antibody of a particular isotype in an antiserum reacts with a given antigen. This reaction is particularly important for detecting small amounts of specific antibodies of the IgE isotype, which are responsible for most allergies. The presence in an individual's serum of IgE binding to an antigen correlates with allergic reactions to that antigen.

An alternative approach to detecting bound antibodies exploits bacterial proteins that bind to immunoglobulins with high affinity and specificity. One of these, **Protein A** from the bacterium *Staphylococcus aureus*, has been exploited widely in immunology for the affinity purification of immunoglobulin and for the detection of bound antibody. The use of standard second reagents such as labeled anti-immunoglobulin antibodies or Protein A to detect antibody bound specifically to its antigen allows great savings in reagent labeling costs, and also provides a standard detection system so that results in different assays can be compared directly.

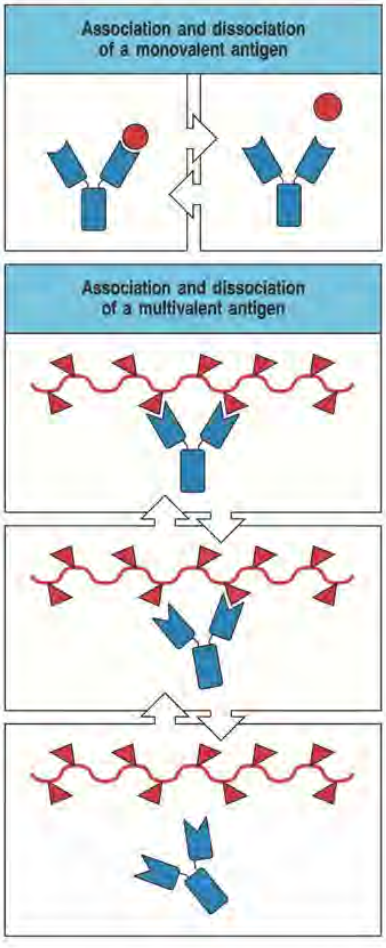


Fig. A.12 The avidity of an antibody is its strength of binding to intact antigen. When an IgG antibody binds a ligand with multiple identical epitopes, both binding sites can bind the same molecule or particle. The overall strength of binding, called avidity, is greater than the affinity, the strength of binding of a single site, because both binding sites must dissociate at the same time for the antibody to release the antigen. This property is very important in the binding of antibody to bacteria, which usually have multiple identical epitopes on their surfaces.

A-11 Coombs tests and the detection of Rhesus incompatibility.

These tests use anti-immunoglobulin antibodies (see Section A-10) to detect the antibodies that cause **hemolytic disease of the newborn**, or **erythroblastosis fetalis**. Anti-immunoglobulin antibodies were first developed by Robin Coombs, and the test for this disease is still called the Coombs test. Hemolytic disease of the newborn occurs when a mother makes IgG antibodies specific for the **Rhesus** or **Rh blood group antigen** expressed on the red blood cells of her fetus. Rh-negative mothers make these antibodies when they are exposed to Rh-positive fetal red blood cells bearing the paternally inherited Rh antigen. Maternal IgG antibodies are normally transported across the placenta to the fetus, where they protect the newborn infant against infection. However, IgG anti-Rh antibodies coat the fetal red blood cells, which are then destroyed by phagocytic cells in the liver, causing a hemolytic anemia in the fetus and newborn infant.

Rh antigens are widely spaced on the red blood cell surface, and so the IgG anti-Rh antibodies do not bind in the correct conformation to fix complement and so do not cause lysis of red blood cells *in vitro*. Furthermore, for reasons that are not fully understood, antibodies against Rh antigens do not agglutinate red blood cells, unlike antibodies against the ABO blood group antigens. Thus, detecting anti-Rh antibodies was difficult until anti-human immunoglobulin antibodies were developed. With these, maternal IgG

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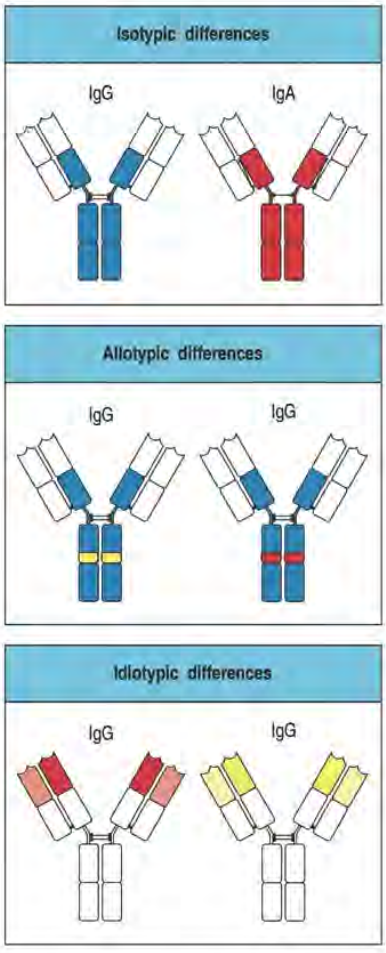


Fig. A.13 Different types of variation between immunoglobulins. Differences between constant regions due to the usage of different C-region genes are called isotypes; differences due to different alleles of the same C gene are called allotypes; differences due to particular rearranged V_H and V_L genes are called idiotypes.

antibodies bound to the fetal red blood cells can be detected after washing the cells to remove unbound immunoglobulin that is present in the fetal serum. Adding anti-human immunoglobulin antibodies against the washed fetal red blood cells agglutinates any cells to which maternal antibodies are bound. This is the **direct Coombs test** (Fig. A.14), so called because it directly detects antibody bound to the surface of the fetal red blood cells. An **indirect Coombs test** is used to detect nonagglutinating anti-Rh antibody in maternal serum: the serum is first incubated with Rh-positive red blood cells, which bind the anti-Rh antibody, after which the antibody-coated cells are washed to remove unbound immunoglobulin and are then agglutinated with anti-immunoglobulin antibody (see Fig. A.14). The indirect Coombs test allows Rh incompatibilities that might lead to hemolytic disease of the newborn to be detected, and this knowledge allows the disease to be prevented (see Section 11-19). The Coombs test is also commonly used to detect antibodies against drugs that bind to red blood cells and cause hemolytic anemia.

A-12 Monoclonal antibodies.

The antibodies generated in a natural immune response or after immunization in the laboratory are a mixture of molecules of different specificities and affinities. Some of this heterogeneity results from the production of antibodies that bind to different epitopes on the immunizing antigen, but even antibodies directed at a single antigenic determinant such as a hapten can be markedly heterogeneous, as shown by **isoelectric focusing.** In this technique, proteins are separated on the basis of their isoelectric point, the pH at which their net charge is zero. By electrophoresing proteins in a pH gradient for long enough, each molecule migrates along the pH gradient until it reaches the pH at which it is neutral and is thus concentrated (focused) at that point. When antiserum containing anti-hapten antibodies is treated in this way and then transferred to a solid support such as nitrocellulose paper, the anti-hapten antibodies can be detected by their ability to bind labeled hapten. The binding of antibodies of various isoelectric points to the hapten shows that even antibodies that bind the same antigenic determinant can be heterogeneous.

Antisera are valuable for many biological purposes but they have certain inherent disadvantages that relate to the heterogeneity of the antibodies they contain. First, each antiserum is different from all other antisera, even if raised in a genetically identical animal by using the identical preparation of antigen and the same immunization protocol. Second, antisera can be produced in only limited volumes, and thus it is impossible to use the identical serological reagent in a long or complex series of experiments or clinical tests. Finally, even antibodies purified by affinity chromatography (see Section A-5) can include minor populations of antibodies that give unexpected cross-reactions, which confound the analysis of experiments. To avoid these problems, and to harness the full potential of antibodies, it was necessary to develop a way of making an unlimited supply of antibody molecules of homogeneous structure and known specificity. This has been achieved through the production of monoclonal antibodies from cultures of hybrid antibody-forming cells or, more recently, by genetic engineering.

Biochemists in search of a homogeneous preparation of antibody that they could subject to detailed chemical analysis turned early to proteins produced by patients with multiple myeloma, a common tumor of plasma cells. It was known that antibodies are normally produced by plasma cells, and because this disease is associated with the presence of large amounts of a homogeneous gamma globulin called a **myeloma protein** in the patient's serum, it seemed likely that myeloma proteins would serve as models for normal antibody molecules. Thus, much of the early knowledge of antibody structure came from studies on myeloma proteins. These studies showed that

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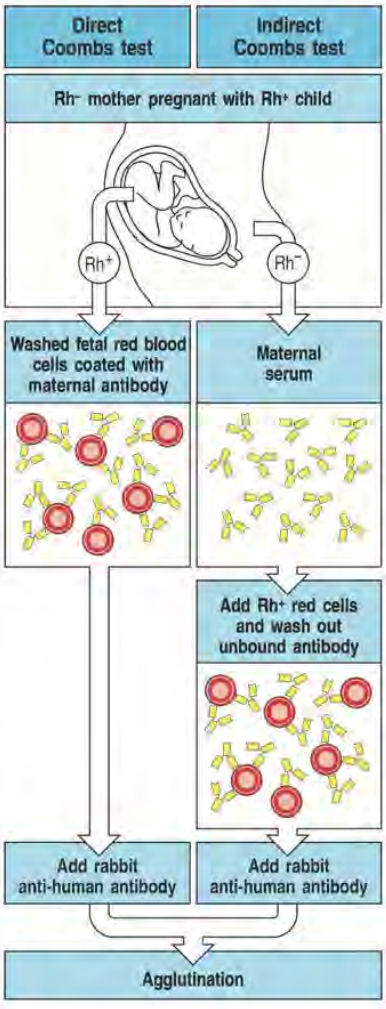


Fig. A.14 The Coombs direct and indirect anti-globulin tests for antibody against red blood cell antigens. A Rh^- mother of a Rh^+ fetus can become immunized to fetal red blood cells that enter the maternal circulation at the time of delivery. In a subsequent pregnancy with a Rh^+ fetus, IgG anti-Rh antibodies can cross the placenta and damage the fetal red blood cells. In contrast to anti-Rh antibodies, maternal anti-ABO antibodies are of the IgM isotype and cannot cross the placenta, and so do not cause harm. Anti-Rh antibodies do not agglutinate red blood cells, but their presence on the fetal red blood cell surface can be shown by washing away unbound immunoglobulin and then adding antibody against human immunoglobulin, which agglutinates the antibody-coated cells. Anti-Rh antibodies can be detected in the mother's serum in an indirect Coombs test; the serum is incubated with Rh^+ red blood cells, and once the antibody has bound, the red blood cells are treated as in the direct Coombs test.

monoclonal antibodies could be obtained from immortalized plasma cells. However, the antigen specificity of most myeloma proteins was unknown, which limited their usefulness as objects of study or as immunological tools.

This problem was solved by Georges Köhler and César Milstein, who devised a technique for producing a homogeneous population of antibodies of known antigenic specificity. They did this by fusing spleen cells from an immunized mouse to cells of a mouse myeloma to produce hybrid cells that both proliferated indefinitely and secreted antibody specific for the antigen used to immunize the spleen cell donor. The spleen cell provides the ability to make specific antibody, while the myeloma cell provides the ability to grow indefinitely in culture and secrete immunoglobulin continuously. By using a myeloma cell partner that produces no antibody proteins itself, the antibody produced by the hybrid cells comes only from the immune spleen cell partner. After fusion, the hybrid cells are selected using drugs that kill the myeloma parental cell, while the unfused parental spleen cells have a limited life-span and soon die, so that only hybrid myeloma cell lines or **hybridomas** survive. Those hybridomas producing antibody of the desired specificity are then identified and cloned by regrowing the cultures from single cells (Fig. A.15). Because each hybridoma is a **clone** derived from fusion with a single B cell, all the antibody molecules it produces are identical in structure, including their antigen-binding site and isotype. Such antibodies are called **monoclonal antibodies**. This technology has revolutionized the use of antibodies by providing a limitless supply of antibody of a single and known specificity. Monoclonal antibodies are now used in most serological assays, as diagnostic probes, and as therapeutic agents. So far, however, only mouse monoclonals are routinely produced in this way, and efforts to use the same approach to make human monoclonal antibodies have met with limited success. 'Fully human' therapeutic monoclonal antibodies are currently made using either the phage display technology described in Section A-13 or transgenic mice carrying human antibody genes.

A-13 Phage display libraries for antibody V-region production.

This is a technique for producing antibody-like molecules. Gene segments encoding the antigen-binding variable, or V, domains of antibodies are fused to genes encoding the coat protein of a bacteriophage. Bacteriophages containing such gene fusions are used to infect bacteria, and the resulting phage particles have coats that express the antibody-like fusion protein, with the antigen-binding domain displayed on the outside of the bacteriophages. A collection of recombinant phages, each displaying a different antigen-binding domain on the surface, is known as a **phage display library**. In much the same way that antibodies specific for a particular antigen can be isolated from a complex mixture by affinity chromatography (see Section A-5), phages expressing antigen-binding domains specific for a particular antigen can be

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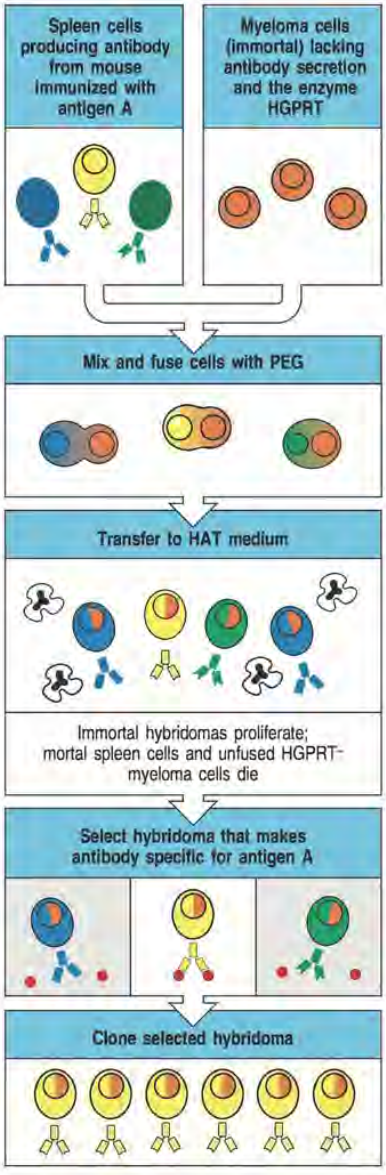


Fig. A.15 The production of monoclonal antibodies. Mice are immunized with antigen A and given an intravenous booster immunization 3 days before they are killed, in order to produce a large population of spleen cells secreting specific antibody. Spleen cells die after a few days in culture. To produce a continuous source of antibody they are fused with immortal myeloma cells by using polyethylene glycol (PEG) to produce a hybrid cell line called a hybridoma. The myeloma cells are selected beforehand to ensure that they are not secreting antibody themselves and that they are sensitive to the hypoxanthine–aminopterin–thymidine (HAT) medium that is used to select hybrid cells because they lack the enzyme hypoxanthine:guanine phosphoribosyl transferase (HGPRT). The HGPRT gene contributed by the spleen cell allows hybrid cells to survive in the HAT medium, and only hybrid cells can grow continuously in culture because of the malignant potential contributed by the myeloma cells. Unfused myeloma cells and unfused spleen cells therefore die in the HAT medium, as shown here by cells with dark, irregular nuclei. Individual hybridomas are then screened for antibody production, and cells that make antibody of the desired specificity are cloned by growing them up from a single antibody-producing cell. The cloned hybridoma cells are grown in bulk culture to produce large amounts of antibody. As each hybridoma is descended from a single cell, all the cells of a hybridoma cell line make the same antibody molecule, which is thus called a monoclonal antibody.

isolated by selecting the phages in the library for binding to that antigen. The phage particles that bind are recovered and used to infect fresh bacteria. Each phage isolated in this way will produce a monoclonal antigen-binding particle analogous to a monoclonal antibody (Fig. A.16). The genes encoding the antigen-binding site, which are unique to each phage, can then be recovered from the phage DNA and used to construct genes for a complete antibody molecule by joining them to parts of immunoglobulin genes that encode the invariant parts of an antibody. When these reconstructed antibody genes are introduced into a suitable host cell line, such as the nonantibody-producing myeloma cells used for hybridomas, the transfected cells can secrete antibodies with all the desirable characteristics of monoclonal antibodies produced from hybridomas.

In much the same way that a collection of phages can display a wide variety of potential antigen-binding sites, the phages can also be engineered to display a wide variety of antigens; such a library is known as an **antigen display library**. In such cases, the antigens displayed are often short peptides encoded by chemically synthesized DNA sequences that have mixtures of all four nucleotides in some positions, so that all possible amino acids are incorporated. It is not usual for every position in a peptide to be allowed to vary in this way, because the number of different peptide sequences increases dramatically with the number of variable positions; there are more than 2×10^{10} possible sequences of eight amino acids.

A-14 Microscopy and imaging.

Because antibodies bind stably and specifically to their corresponding antigen, they are invaluable as probes for identifying a particular molecule in cells, tissues, or biological fluids. Antibody molecules can be used to locate their target molecules accurately in single cells or in tissue sections by a variety of different labeling techniques. When the antibody itself, or the anti-immunoglobulin antibody used to detect it, is labeled with a fluorescent dye (a fluorochrome or fluorophore), the technique is known as **immunofluorescence microscopy**. As in all serological techniques, the antibody binds stably to its antigen, allowing unbound antibody to be removed by thorough washing. Because antibodies against proteins recognize the surface features of the native, folded protein, the native structure of the protein being sought usually needs to be preserved, either by using only the most gentle chemical fixation techniques or by using frozen tissue sections that are fixed only after the antibody reaction has been performed. Some antibodies, however, bind proteins

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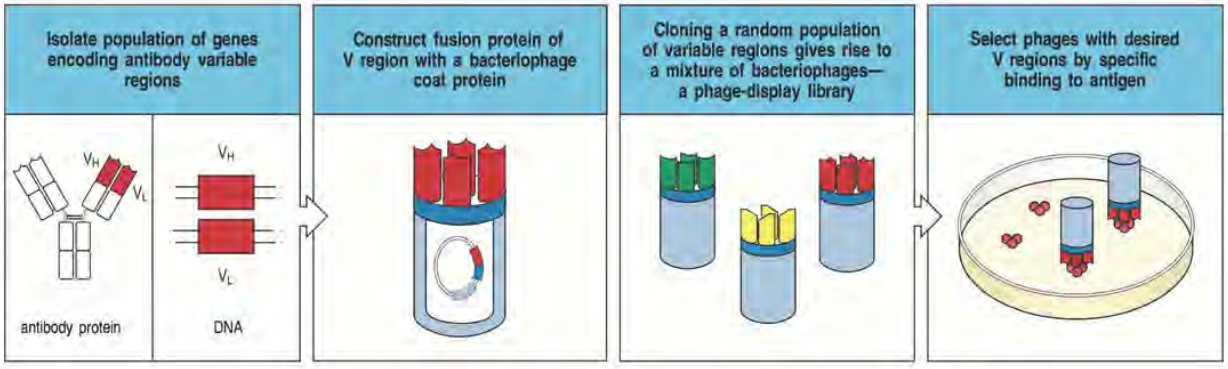


Fig. A.16 The production of antibodies by genetic engineering. Short primers to consensus sequences in heavy- and light-chain variable (V) regions of immunoglobulin genes are used to generate a library of heavy- and light-chain V-region DNAs by PCR, with spleen DNA as the starting material. These heavy- and light-chain V-region genes are cloned randomly into filamentous phages such that each phage expresses one heavy-chain and one light-chain V region as a surface fusion protein with antibody-like properties. The resulting phage display library is multiplied in bacteria, and the phages are then bound to a surface coated with antigen. The unbound phages are washed away; the bound phages are recovered, multiplied in bacteria, and again bound to antigen. After a few cycles, only specific high-affinity antigen-binding phages are left. These can be used like antibody molecules, or their V genes can be recovered and engineered into antibody genes to produce genetically engineered antibody molecules (not shown). This technology may replace the hybridoma technology for producing monoclonal antibodies, and has the advantage that humans can be used as the source of DNA.

even if they are denatured, and such antibodies will bind specifically even to protein in fixed tissue sections.

The fluorescent dye can be covalently attached directly to the specific antibody; however, the bound antibody is more commonly detected by fluorescently labeled anti-immunoglobulin, a technique known as **indirect immunofluorescence**. The dyes chosen for immunofluorescence are excited by light of one wavelength, usually blue or green, and emit light of a different wavelength in the visible spectrum. The most commonly used fluorochromes are fluorescein, which emits green light, Texas Red and Peridinin chlorophyll protein (PerCP), which emit red light, and rhodamine and phycoerythrin (PE) which emit orange/red light (Fig. A.17). By using selective filters, only the light coming from the fluorochrome used is detected in the fluorescence microscope (Fig. A.18). Although Albert Coons first devised this technique to identify the plasma cell as the source of antibody, it can be used to detect the distribution of any protein. By attaching different dyes to different antibodies, the distribution of two or more molecules can be determined in the same cell or tissue section (see Fig. A.18).

The development of the **confocal fluorescent microscope**, which uses computer-aided techniques to produce ultrathin optical sections of a cell or tissue, gives very high resolution (sub-micrometer) fluorescence microscopy without the need for elaborate sample preparation. The light source for excitation (a laser) is focused onto a particular plane in the specimen, and the emitted light is refocused through a 'pinhole' so that only light from the desired plane reaches the detector, thus removing out-of-focus emissions from above or below the plane. This gives a sharper image than conventional fluorescence microscopy, and a three-dimensional picture can be built up from successive

Excitation and emission wavelengths of some commonly used fluorochromes

Probe	Excitation (nm)	Emission (nm)
R-phycoerythrin (PE)	480; 565	578
Fluorescein	495	519
PerCP	490	675
Texas Red	589	615
Rhodamine	550	573

Fig. A.17 Excitation and emission wavelengths for common fluorochromes.

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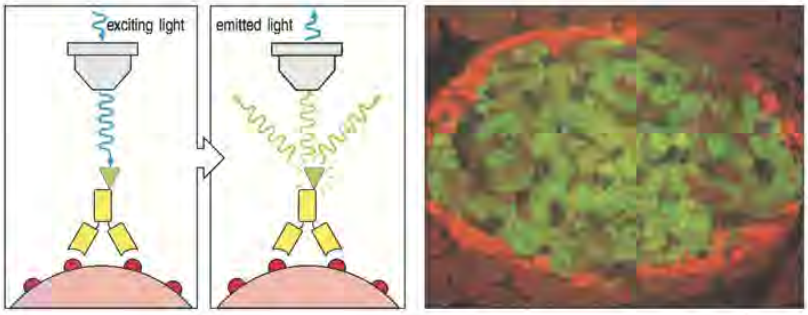


Fig. A.18 Immunofluorescence microscopy. Antibodies labeled with a fluorescent dye such as fluorescein (green triangle) are used to reveal the presence of their corresponding antigens in cells or tissues. The stained cells are examined in a microscope that exposes them to blue or green light to excite the fluorescent dye. The excited dye emits light at a characteristic wavelength, which is captured by viewing the sample through a selective filter. This technique is applied widely in biology to determine the location of molecules in cells and tissues. Different antigens can be detected in tissue sections by labeling antibodies with dyes of distinctive color. Here, antibodies against the protein glutamic acid decarboxylase (GAD) coupled to a green dye are shown to stain the β cells of pancreatic islets of Langerhans. The α cells do not make this enzyme and are labeled with antibodies against the hormone glucagon coupled with an orange fluorescent dye. GAD is an important antigen in diabetes, a disease in which the insulin-secreting β cells of the islets of Langerhans are destroyed by an immune attack on self tissues (see Chapter 15). Photograph courtesy of M. Solimena and P. De Camilli.

optical sections taken along the 'vertical' axis. Confocal microscopy can be used on fixed cells stained with fluorescently tagged antibodies or on living cells expressing proteins tagged with naturally fluorescent proteins. The first of these fluorescent proteins to come into wide use was green fluorescent protein (GFP), isolated from the jellyfish *Aequorea victoria*. The list of fluorescent proteins in routine use now includes those emitting red, blue, cyan, or yellow fluorescence. By using cells transfected with genes encoding different fusion proteins, it has been possible to visualize the redistribution of T-cell receptors, co-receptors, adhesion molecules, and other signaling molecules, such as CD45, that takes place when a T cell makes contact with a target cell (see Fig. 9.31).

Confocal microscopy, however, can only penetrate around 80 μm into a tissue, and at the wavelengths typically used for excitation, the source light will soon bleach the fluorescent label and damage the specimen. This means that the technique is not suitable for imaging a live specimen over a period of time sufficient, for example, to track the movements of cells in a tissue. The more recently developed technique of **two-photon scanning fluorescence microscopy** overcomes some of these limitations. Like confocal microscopy, two-photon microscopy produces thin optical sections from which a three-dimensional image can be built up. However, in this case, ultrashort pulses of laser light of much longer wavelength (and thus with photons of lower energy) are used for excitation, and two of these lower-energy photons arriving nearly simultaneously are required to excite the fluorophore. Excitation will therefore only occur in a very small region at the focus of the microscope, where the beam of light is most intense, and so fluorescence emission will be restricted to the plane of focus, producing a sharp, high-contrast image. The longer-wavelength light (typically in the near infrared) is also less damaging to living tissue than the blue and ultraviolet wavelengths typically used in confocal microscopy, and so imaging can be carried out over a longer period. More of the emitted light is collected than in confocal microscopy, and because single photons scattering within the tissue cannot cause fluorescence and consequent background haze, imaging to greater depths (several hundred micrometers) is possible.

To track the movements of molecules or cells over time, confocal or two-photon microscopy is combined with **time-lapse video imaging** using sensitive digital cameras. In immunology, time-lapse two-photon fluorescence imaging has been particularly valuable for tracking the movements of individual T cells and B cells expressing fluorescent proteins in intact lymphoid organs and observing where they interact (see Chapter 10).

A-15 Immunoelectron microscopy.

Antibodies can be used to detect the intracellular location of structures or particular proteins at high resolution by electron microscopy, a technique

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known as **immunoelectron microscopy**. Antibodies against the required antigen are labeled with gold particles and then applied to ultrathin sections, which are then examined in the transmission electron microscope. Antibodies labeled with gold particles of different diameters enable two or more proteins to be studied simultaneously (see Fig. 6.11). The difficulty with this technique is in staining the ultrathin section adequately, because few molecules of antigen are present in each section.

A-16 Immunohistochemistry.

An alternative to immunofluorescence (see Section A-14) for detecting a protein in tissue sections is **immunohistochemistry**, in which the specific antibody is chemically coupled to an enzyme that converts a colorless substrate into a colored reaction product *in situ*. The localized deposition of the colored product where antibody has bound can be directly observed under a light microscope. The antibody binds stably to its antigen, allowing unbound antibody to be removed by thorough washing. This method of detecting bound antibody is analogous to ELISA (see Section A-6) and frequently uses the same coupled enzymes, the difference in detection being primarily that in immunohistochemistry the colored products are insoluble and precipitate at the site where they are formed. Horseradish peroxidase and alkaline phosphatase are the two enzymes most commonly used in these applications. Horseradish peroxidase oxidizes the substrate diaminobenzidine to produce a brown precipitate, while alkaline phosphatase can produce red or blue dyes depending on the substrates used; a common substrate is 5-bromo-4-chloro-3-indolyl phosphate plus nitroblue tetrazolium (BCIP/NBT), which gives rise to a dark blue or purple stain. As with immunofluorescence, the native structure of the protein being sought usually needs to be preserved so that it will be recognized by the antibody. Tissues are fixed by the most gentle chemical fixation techniques, or frozen tissue sections are used that are fixed only after the antibody reaction has been performed.

A-17 Immunoprecipitation and co-immunoprecipitation.

To raise antibodies against membrane proteins and other cellular structures that are difficult to purify, mice are often immunized with whole cells or crude cell extracts. Antibodies against the individual molecules are then obtained by using these immunized mice to produce hybridomas making monoclonal antibodies (see Section A-12) that bind to the cell type used for immunization. To characterize the molecules identified by the antibodies, cells of the same type are labeled with radioisotopes and dissolved in nonionic detergents that disrupt cell membranes but do not interfere with antigen–antibody interactions. This allows the labeled protein to be isolated by binding to the antibody in a reaction known as **immunoprecipitation**. The antibody is usually attached to a solid support, such as the beads that are used in affinity chromatography (see Section A-5), or to Protein A. Cells can be labeled in two main ways for immunoprecipitation analysis. All the proteins in a cell can be labeled metabolically by growing the cell in radioactive amino acids that are incorporated into cellular protein (Fig. A.19). Alternatively, one can label only the cell-surface proteins by radioiodination under conditions that prevent iodine from crossing the plasma membrane and labeling proteins inside the cell, or by a reaction that labels only membrane proteins with biotin, a small molecule that is detected readily by labeled avidin, a protein found in egg whites that binds biotin with very high affinity.

Once the labeled proteins have been isolated by the antibody, they can be characterized in several ways. The most common is polyacrylamide gel electrophoresis (PAGE) of the proteins after they have been dissociated from

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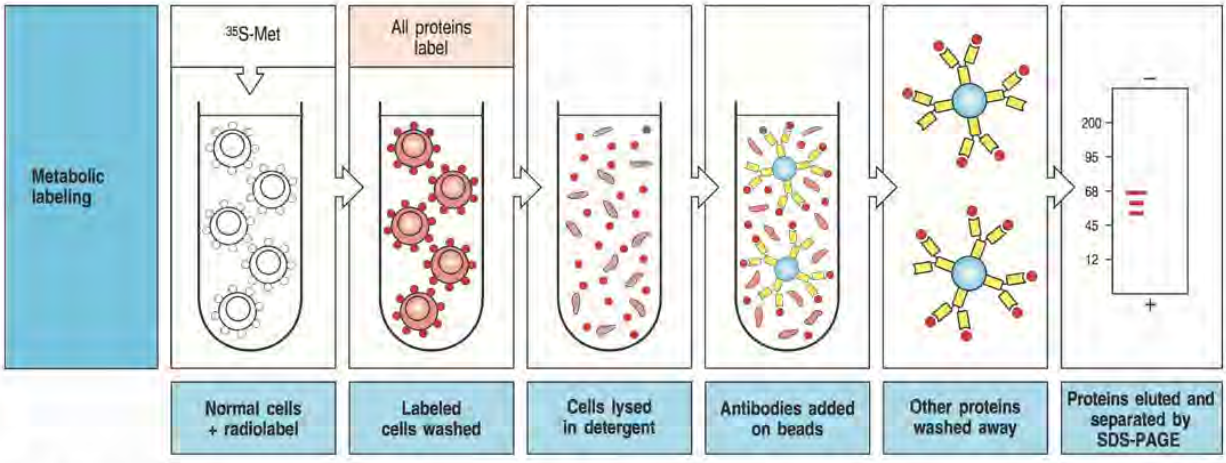


Fig. A.19 Cellular proteins reacting with an antibody can be characterized by immunoprecipitation of labeled cell lysates. All actively synthesized cellular proteins can be labeled metabolically by incubating cells with radioactive amino acids (shown here for methionine; ^{35}S-Met), or one can label just the cell-surface proteins by using radioactive iodine in a form that cannot cross the cell membrane or by a reaction with the small molecule biotin, detected by its reaction with labeled avidin (not shown). Cells are lysed with detergent and individual labeled cell-associated proteins can be precipitated with a monoclonal antibody attached to beads. After unbound proteins have been washed away, the bound protein is eluted in the detergent sodium dodecyl sulfate (SDS), which dissociates it from the antibody and also coats the protein with a strong negative charge, allowing it to migrate according to its size in polyacrylamide gel electrophoresis (PAGE). The positions of the labeled proteins are determined by autoradiography using X-ray film. This technique of SDS-PAGE can be used to determine the molecular weight and subunit composition of a protein. Patterns of protein bands observed with metabolic labeling are usually more complex than those revealed by radioiodination, owing to the presence of precursor forms of the protein (right panel). The mature form of a surface protein can be identified as being the same size as that detected by surface iodination or biotinylation (not shown).

antibody in the strong ionic detergent sodium dodecyl sulfate (SDS), a technique generally abbreviated as **SDS-PAGE**. SDS binds relatively homogeneously to proteins, conferring a charge that allows the electrophoretic field to drive protein migration through the gel. The rate of migration is controlled mainly by protein size (see Fig. A.19). Proteins of differing charges can be separated using isoelectric focusing (see Section A-12). This technique can be combined with SDS-PAGE in a procedure known as **two-dimensional gel electrophoresis**. For this, the immunoprecipitated protein is eluted in urea, a nonionic solubilizing agent, and run on an isoelectric focusing gel in a narrow tube of polyacrylamide. This first-dimensional isoelectric focusing gel is then placed across the top of an SDS-PAGE slab gel, which is then run vertically to separate the proteins by molecular weight (Fig. A.20). Two-dimensional gel electrophoresis is a powerful technique that allows many hundreds of proteins in a complex mixture to be distinguished from one another.

Immunoprecipitation and the related technique of immunoblotting (see Section A-18) are useful for determining the molecular weight and isoelectric point of a protein as well as its abundance, distribution, and whether, for

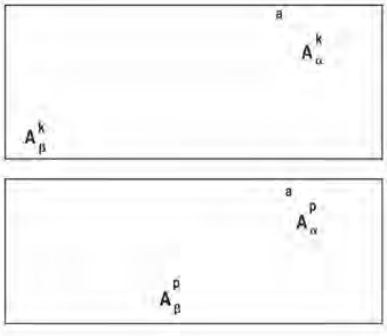


Fig. A.20 Two-dimensional gel electrophoresis of MHC class II molecules. Proteins in mouse spleen cells have been labeled metabolically (see Fig. A.19), precipitated with a monoclonal antibody against the mouse MHC class II molecule H2-A, and separated by isoelectric focusing in one direction and SDS-PAGE in a second direction at right angles to the first (hence the term two-dimensional gel electrophoresis). This allows one to distinguish molecules of the same molecular weight on the basis of their charge. The separated proteins are detected by autoradiography. The MHC class II molecules are composed of two chains, α and β, and in the different MHC class II molecules these have different isoelectric points (compare upper and lower panels). The MHC genotype of mice is indicated by lower-case superscripts (k, p). Actin, a common contaminant, is marked a. Photographs courtesy of J.F. Babich.

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example, it undergoes changes in molecular weight and isoelectric point as a result of processing within the cell.

A-18 Immunoblotting (Western blotting).

Like immunoprecipitation (see Section A-17), **immunoblotting** is used for identifying the presence of a given protein in a cell lysate, but it avoids the problem of having to label large quantities of cells with radioisotopes. Unlabeled cells are placed in detergent to solubilize all cell proteins, and the lysate is run on SDS-PAGE to separate the proteins (see Section A-17). The size-separated proteins are then transferred from the gel to a stable support such as a nitrocellulose membrane. Specific proteins are detected by treatment with antibodies able to react with SDS-solubilized proteins (mainly those that react with denatured sequences); the bound antibodies are revealed by anti-immunoglobulin antibodies labeled with radioisotopes or an enzyme. The term **Western blotting** as a synonym for immunoblotting arose because the comparable technique for detecting specific DNA sequences is known as Southern blotting, after Ed Southern who devised it, which in turn provoked the name Northern for blots of size-separated RNA, and Western for blots of size-separated proteins. Western blots have many applications in basic research and clinical diagnosis. They are often used to test sera for the presence of antibodies against specific proteins, for example to detect antibodies against different constituents of HIV (Fig. A.21).

Co-immunoprecipitation is an extension of the immunoprecipitation technique that is used to determine whether a given protein interacts physically with another given protein. Cell extracts containing the presumed interaction complex are first immunoprecipitated with antibody against one of the proteins. The material identified by this means is then tested for the presence of the other protein by immunoblotting with a specific antibody.

A-19 Use of antibodies in the isolation and identification of genes and their products.

As a first step in isolating the gene that codes for a particular protein, antibodies specific for the protein can be used to isolate the purified protein from cells by affinity chromatography (see Section A-5). Small amounts of amino acid sequence can then be obtained from the protein's amino-terminal end or from peptide fragments generated by proteolysis. The information in these amino acid sequences is used to construct a set of synthetic oligonucleotides corresponding to the possible DNA sequences, which are then used as probes to isolate the gene encoding the protein from either a library of DNA sequences complementary to mRNA (a cDNA library) or a genomic DNA library (a library of chromosomal DNA fragments).

An alternative approach to gene identification uses antibodies to identify the protein produced by a gene that has been introduced into a cell that does not normally express it. This technique is most commonly applied to the

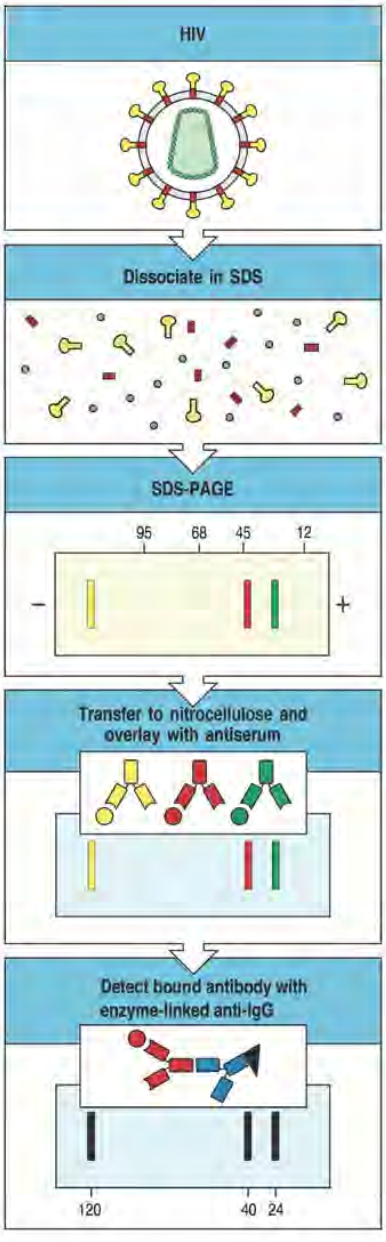


Fig. A.21 Western blotting is used to identify antibodies against the human immunodeficiency virus (HIV) in serum from infected individuals. The virus is dissociated into its constituent proteins by treatment with the detergent SDS, and its proteins are separated using SDS-PAGE. The separated proteins are transferred to a nitrocellulose sheet and reacted with the test serum. Anti-HIV antibodies in the serum bind to the various HIV proteins and are detected with enzyme-linked anti-human immunoglobulin, which deposits colored material from a colorless substrate. This general methodology will detect any combination of antibody and antigen and is used widely, although the denaturing effect of SDS means that the technique works most reliably with antibodies that recognize the antigen when it is denatured.

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identification of genes encoding cell-surface proteins. A set of cDNA-containing expression vectors is first made from a cDNA library prepared from the total mRNA from a cell type that does express the protein of interest. The vectors are used to transfect a cell type that does not normally express the protein of interest, and the vector drives expression of the cDNA it contains without integrating into the host cell DNA. Cells expressing the required protein after transfection are then isolated by binding to specific antibodies that detect the presence of the protein on the cell surface. The vector containing the required gene can then be recovered from these cells (Fig. A.22).

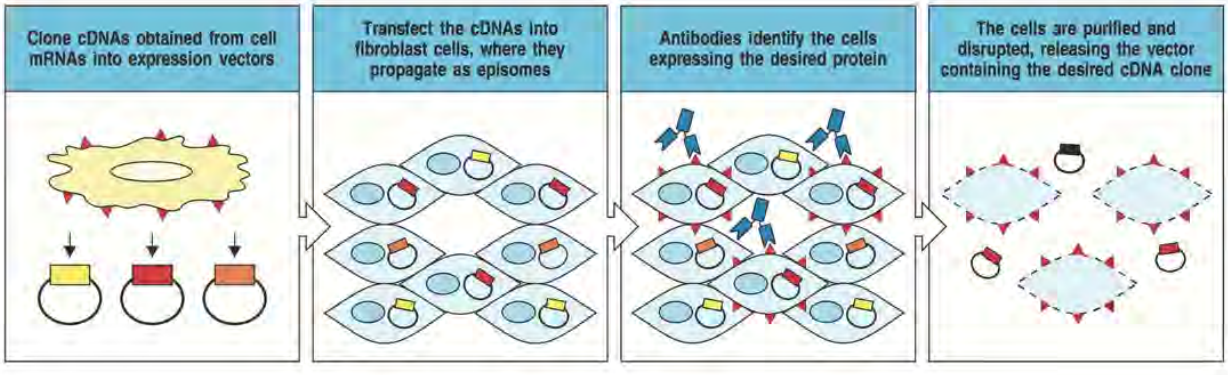
The recovered vectors are then introduced into bacterial cells, where they replicate rapidly; these amplified vectors are used in a second round of transfection in mammalian cells. After several cycles of transfection, isolation, and amplification in bacteria, single colonies of bacteria are picked and the vectors prepared from cultures of each colony are used in a final transfection to identify a cloned vector carrying the cDNA of interest, which is then isolated and characterized. This methodology has been used to isolate many genes encoding cell-surface molecules.

The full amino acid sequence of the protein can be deduced from the nucleotide sequence of its cDNA, and this often gives clues to the nature of the protein and its biological properties. The nucleotide sequence of the gene and its regulatory regions can be determined from genomic DNA clones. The gene can be manipulated and introduced into cells by transfection for larger-scale production and functional studies. This approach has been used to characterize many immunologically important proteins, such as the MHC glycoproteins.

The converse approach is taken to identify the unknown protein product of a cloned gene. The gene sequence is used to construct synthetic peptides of 10–20 amino acids that are identical to part of the deduced protein sequence, and antibodies are then raised against these peptides by coupling them to carrier proteins; the peptides behave as haptens. These anti-peptide antibodies often bind the native protein and so can be used to identify its distribution in cells and tissues and to try to ascertain its function (Fig. A.23). This approach to identifying the function of a gene is often called 'reverse genetics' because it works from gene to phenotype rather than from phenotype to gene, which is the classical genetic approach. The great advantage of reverse genetics over the classical approach is that it does not require a detectable phenotypic genetic trait in order to identify a gene.

Antibodies can also be used in the determination of the function of gene products. Some antibodies are able to act as agonists, when the binding of the antibody to the molecule mimics the binding of the natural ligand and activates the function of the gene product. For example, antibodies against the CD3 molecule have been used to stimulate T cells, replacing the interaction of the

Fig. A.22 The gene encoding a cell-surface molecule can be isolated by expressing it in fibroblasts and detecting its protein product with monoclonal antibodies. Total mRNA from a cell line or tissue expressing the protein is isolated, converted into cDNA, and cloned as cDNAs in a vector designed to direct expression of the cDNA in fibroblasts. The entire cDNA library is used to transfect cultured fibroblasts. Fibroblasts that have taken up cDNA encoding a cell-surface protein express the protein on their surface; they can be isolated by binding a monoclonal antibody against that protein. The vector containing the gene is isolated from the cells that express the antigen and used for more rounds of transfection and reisolation until uniform positive expression is obtained, ensuring that the correct gene has been isolated. The cDNA insert can then be sequenced to determine the sequence of the protein it encodes and can also be used as the source of material for large-scale expression of the protein for analysis of its structure and function. The method illustrated is limited to cloning genes for single-chain proteins (that is, those encoded by only one gene) that can be expressed in fibroblasts. It has been used to clone many genes of immunological interest such as that for CD4.



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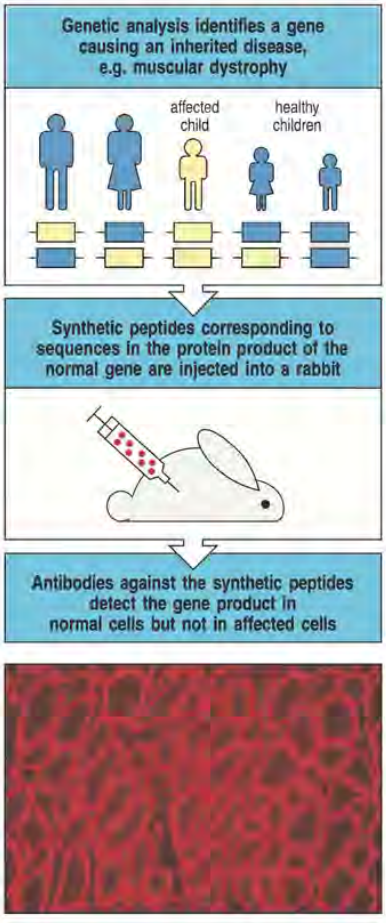


Fig. A.23 The use of antibodies to detect the unknown protein product of a known gene is called reverse genetics. When the gene responsible for a genetic disorder such as Duchenne muscular dystrophy is isolated, the amino acid sequence of the unknown protein product of the gene can be deduced from the nucleotide sequence of the gene, and synthetic peptides representing parts of this sequence can be made. Antibodies are raised against these peptides and purified from the antiserum by affinity chromatography on a peptide column (see Fig. A.5). Labeled antibody is used to stain tissue from individuals with the disease and from unaffected individuals to determine differences in the presence, amount, and distribution of the normal gene product. The product of the dystrophin gene is present in normal mouse skeletal muscle cells, as shown in the bottom panel (red fluorescence), but is missing from the cells of mice bearing the mutation *mdx*, the mouse equivalent of Duchenne muscular dystrophy (not shown). Photograph (×15) courtesy of H.G.W. Lidov and L. Kunkel.

T-cell receptor with peptide:MHC antigens in cases where the specific peptide antigen is not known. Conversely, antibodies can function as antagonists, inhibiting the binding of the natural ligand and thus blocking the function.

Isolation of lymphocytes.

A-20 Isolation of peripheral blood lymphocytes by Ficoll-Hypaque™ gradient.

The first step in studying lymphocytes is to isolate them so that their behavior can be analyzed *in vitro*. Human lymphocytes can be isolated most readily from peripheral blood by density centrifugation over a step gradient consisting of a mixture of the carbohydrate polymer Ficoll-Hypaque™ and the dense iodine-containing compound metrizamide. This yields a population of mononuclear cells at the interface that has been depleted of red blood cells and most polymorphonuclear leukocytes or granulocytes (Fig. A.24). The resulting population, called **peripheral blood mononuclear cells** (**PBMCs**), consists mainly of lymphocytes and monocytes. Although this population is readily accessible, it is not necessarily representative of the lymphoid system, because only recirculating lymphocytes can be isolated from blood.

A particular cell population can be isolated from a sample or culture by binding to antibody-coated plastic surfaces, a technique known as **panning**, or by removing unwanted cells by treatment with specific antibody and complement to kill them. Cells can also be passed over columns of antibody-coated,

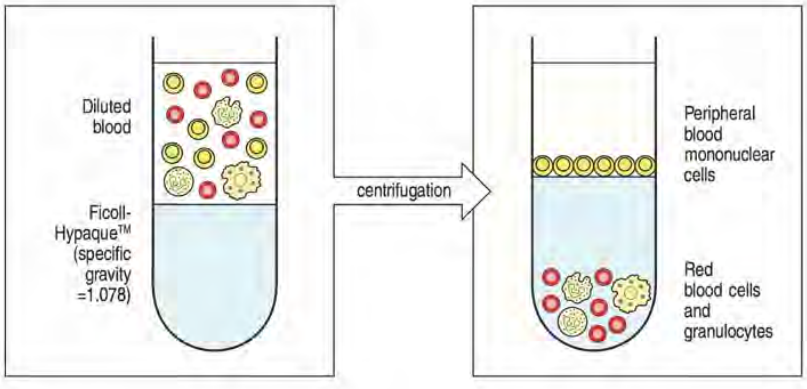


Fig. A.24 Peripheral blood mononuclear cells can be isolated from whole blood by Ficoll-Hypaque™ centrifugation. Diluted anticoagulated blood (left panel) is layered over Ficoll-Hypaque™ and centrifuged. Red blood cells and polymorphonuclear leukocytes or granulocytes are denser and travel through the Ficoll-Hypaque™, while mononuclear cells consisting of lymphocytes together with some monocytes band over it and can be recovered at the interface (right panel).

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nylon-coated steel wool and different populations differentially eluted. This technique extends affinity chromatography to cells, and is now a very popular way to separate cells. All these techniques can also be used as a pre-purification step before sorting out highly purified populations by FACS (see Section A-22).

The 'normal' range of the numbers of the different types of white blood cells in blood, along with the normal ranges of concentrations of the various antibody classes, are given in Fig. A.42.

A-21 Isolation of lymphocytes from tissues other than blood.

In experimental animals, and occasionally in humans, lymphocytes are isolated from lymphoid organs, such as spleen, thymus, bone marrow, lymph nodes, or mucosal-associated lymphoid tissues, most commonly the palatine tonsils in humans (see Fig. 12.6). A specialized population of lymphocytes resides in surface epithelia; these cells are isolated by fractionating the epithelial layer after its detachment from the basement membrane. Finally, in situations where local immune responses are prominent, lymphocytes can be isolated from the site of the response itself. For example, in order to study the autoimmune reaction that is thought to be responsible for rheumatoid arthritis, an inflammatory response in joints, lymphocytes are isolated from the fluid aspirated from the inflamed joint space.

A-22 Flow cytometry and FACS analysis.

Resting lymphocytes are small round cells with a dense nucleus and little cytoplasm (see Fig. 1.7). However, these cells comprise many functional subpopulations, which are usually identified and distinguished from each other on the basis of their differential expression of cell-surface proteins, which can be detected with specific antibodies (Fig. A.25). B and T lymphocytes, for example, are identified unambiguously and separated from each other by antibodies against the constant regions of the B- and T-cell antigen receptors. T cells are further subdivided on the basis of expression of the co-receptor proteins CD4 and CD8.

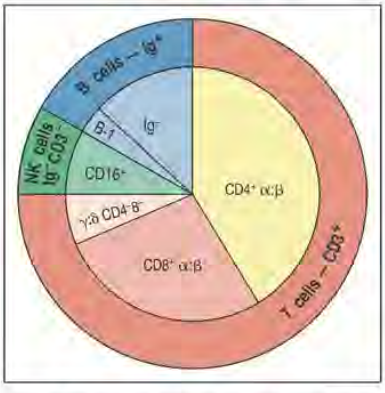


Fig. A.25 The distribution of lymphocyte subpopulations in human peripheral blood. As shown on the outside of the circle, lymphocytes can be divided into T cells bearing T-cell receptors (detected with anti-CD3 antibodies), B cells bearing immunoglobulin receptors (detected with anti-immunoglobulin antibodies), and null cells including natural killer (NK) cells, that label with neither. Further divisions of the T-cell and B-cell populations are shown inside. Using anti-CD4 and anti-CD8 antibodies, α:β T cells can be subdivided into two populations, whereas γ:δ T cells are identified with antibodies against the γ:δ T-cell receptor and mainly lack CD4 and CD8. A minority population of B cells, the so-called B-1 cells, have different characteristics from the majority population (B-2 cells) (see Section 3-24).

An immensely powerful tool for defining and enumerating lymphocytes is the flow cytometer, which detects and counts individual cells passing in a stream through a laser beam. A flow cytometer equipped to separate the identified cells is called a **fluorescence-activated cell sorter** (**FACS**). These instruments are used to study the properties of cell subsets identified using monoclonal antibodies against cell-surface proteins. Individual cells within a mixed population are first tagged by treatment with specific monoclonal antibodies labeled with fluorescent dyes, or by specific antibodies followed by labeled anti-immunoglobulin antibodies. The mixture of labeled cells is then forced with a much larger volume of saline through a nozzle, creating a fine stream of liquid containing cells spaced singly at intervals. As each cell passes through a laser beam it scatters the laser light, and any dye molecules bound to the cell will be excited and will fluoresce. Sensitive photomultiplier tubes detect both the scattered light, which gives information on the size and granularity of the cell, and the fluorescence emissions, which give information on the binding of the labeled monoclonal antibodies and hence on the expression of cell-surface proteins by each cell (Fig. A.26).

In the cell sorter, the signals passed back to the computer are used to generate an electric charge, which is passed from the nozzle through the liquid stream at the precise time that the stream breaks up into droplets, each containing no more than a single cell; droplets containing a charge can then be deflected from the main stream of droplets as they pass between plates of

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opposite charge, so that positively charged droplets are attracted to a negatively charged plate, and vice versa. In this way, specific subpopulations of cells, distinguished by the binding of the labeled antibody, can be purified from a mixed population of cells. Alternatively, to deplete a population of cells, the same fluorochrome can be used to label different antibodies

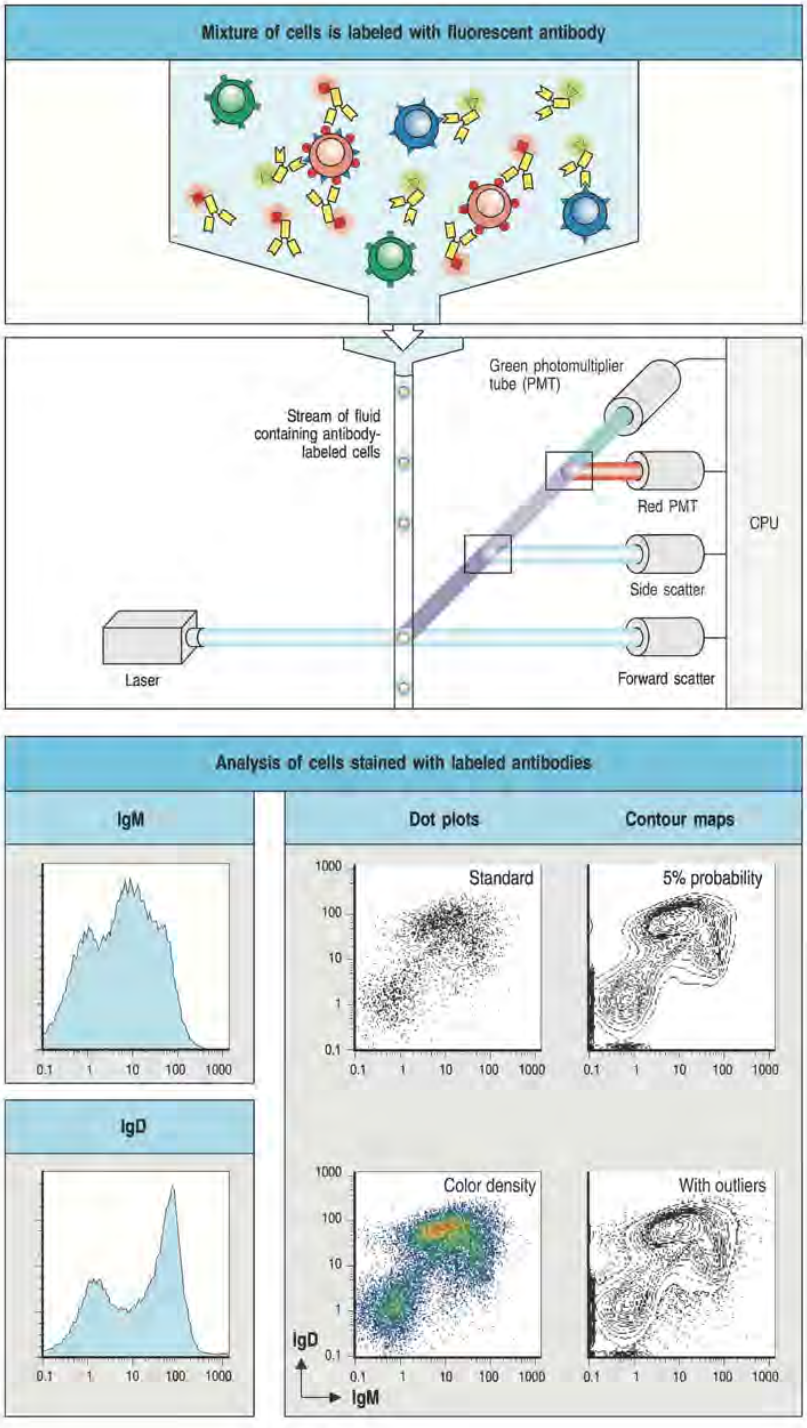
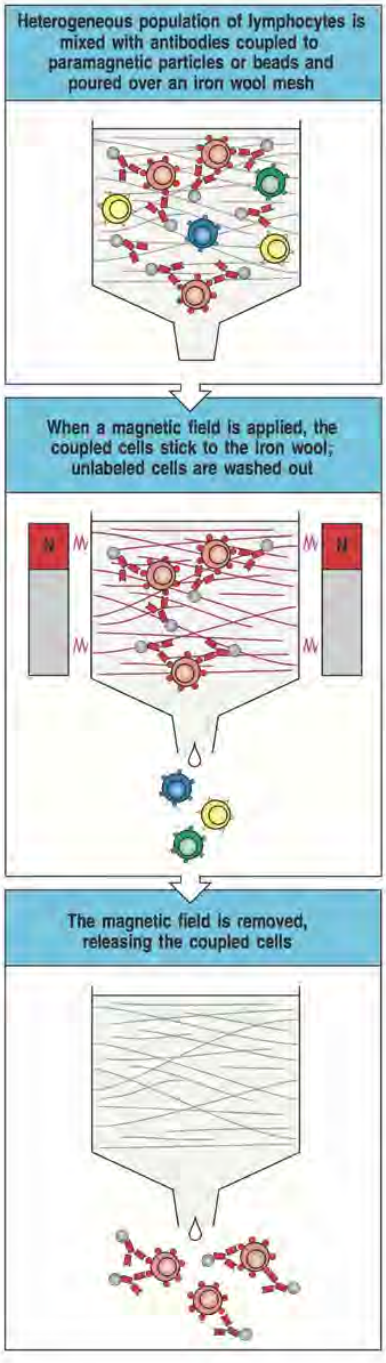


Fig. A.26 The FACS™ allows individual cells to be identified by their cell-surface antigens and to be sorted. Cells to be analyzed by flow cytometry are first labeled with fluorescent dyes (top panel). Direct labeling uses dye-coupled antibodies specific for cell-surface antigens (as shown here), while indirect labeling uses a dye-coupled immunoglobulin to detect unlabeled cell-bound antibody. The cells are forced through a nozzle in a single-cell stream that passes through a laser beam (second panel). Photomultiplier tubes (PMTs) detect the scattering of light, which is a sign of cell size and granularity, and emissions from the different fluorescent dyes. This information is analyzed by computer (CPU). By examining many cells in this way, the number of cells with a specific set of characteristics can be counted and levels of expression of various molecules on these cells can be measured. The lower part of the figure shows how these data can be represented, using the expression of two surface immunoglobulins, IgM and IgD, on a sample of B cells from a mouse spleen. The two immunoglobulins have been labeled with different colored dyes. When the expression of just one type of molecule is to be analyzed (IgM or IgD), the data are usually displayed as a histogram, as in the left-hand panels. Histograms display the distribution of cells expressing a single measured parameter (for example, size, granularity, fluorescence intensity). When two or more parameters are measured for each cell (IgM and IgD), various types of two-color plots can be used to display the data, as shown in the right-hand panel. All four plots represent the same data. The horizontal axis represents intensity of IgM fluorescence, and the vertical axis the intensity of IgD fluorescence. Two-color plots provide more information than histograms; they allow recognition, for example, of cells that are 'bright' for both colors, 'dull' for one and bright for the other, dull for both, negative for both, and so on. For example, the cluster of dots in the extreme lower left portions of the plots represents cells that do not express either immunoglobulin, and are mostly T cells. The standard dot plot (upper left) places a single dot for each cell whose fluorescence is measured. It is good for picking up cells that lie outside the main groups but tends to saturate in areas containing a large number of cells of the same type. A second means of presenting these data is the color dot plot (lower left), which uses color density to indicate high-density areas. A contour plot (upper right) draws 5% 'probability' contours, with 5% of the cells lying between each contour providing the best monochrome visualization of regions of high and low density. The lower right plot is a 5% probability contour map, which also shows outlying cells as dots.

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directed at marker proteins expressed by the various undesired cell types. The cell sorter can be used to direct labeled cells to a waste channel, retaining only the unlabeled cells.

When cells are labeled with a single fluorescent antibody, the data from a flow cytometer are usually displayed in the form of a histogram of fluorescence intensity versus cell numbers. If two or more antibodies are used, each coupled to different fluorescent dyes, then the data are more usually displayed in the form of a two-dimensional scatter diagram or as a contour diagram, where the fluorescence of one dye-labeled antibody is plotted against that of a second, with the result that a population of cells labeling with one antibody can be further subdivided by its labeling with the second antibody (see Fig. A.26). By examining large numbers of cells, flow cytometry can give quantitative data on the percentage of cells bearing different proteins, such as surface immunoglobulin, which characterizes B cells, the T-cell receptor-associated molecules known as CD3, and the CD4 and CD8 co-receptor proteins that distinguish the major T-cell subsets. Likewise, FACS analysis has been instrumental in defining stages in the early development of B and T cells. As the power of the FACS technology has grown, progressively more antibodies labeled with distinct fluorescent dyes can be used at the same time. Three-, four-, and even five-color analyses can now be handled by very powerful machines. FACS analysis has been applied to a broad range of problems in immunology; indeed, it played a vital role in the early identification of AIDS as a disease in which T cells bearing CD4 are depleted selectively (see Chapter 13).



A-23 Lymphocyte isolation using antibody-coated magnetic beads.

Although FACS is superb for isolating small numbers of cells in pure form, when large numbers of lymphocytes must be prepared quickly, mechanical means of separating cells are preferable. A powerful and efficient way of isolating lymphocyte populations is to couple paramagnetic beads to monoclonal antibodies that recognize distinguishing cell-surface molecules. These antibody-coated beads are mixed with the cells to be separated, and run through a column containing material that attracts the paramagnetic beads when the column is placed in a strong magnetic field. Cells binding the magnetically labeled antibodies are retained; cells lacking the appropriate surface molecule can be washed away (Fig. A.27). The bound cells are positively selected for expression of the particular cell-surface molecule, while the unbound cells are negatively selected for its absence.

A-24 Isolation of homogeneous T-cell lines.

The analysis of specificity and effector function in T cells depends heavily on the study of monoclonal populations of T lymphocytes. These can be obtained in four distinct ways. First, by analogy with B-cell hybridomas (see Section A-12), normal T cells proliferating in response to specific antigen can be fused to malignant T-cell lymphoma lines to generate **T-cell hybrids**. The

Fig. A.27 Lymphocyte subpopulations can be separated physically by using antibodies coupled to paramagnetic particles or beads. A mouse monoclonal antibody specific for a particular cell-surface molecule is coupled to paramagnetic particles or beads. It is mixed with a heterogeneous population of lymphocytes and poured over an iron wool mesh in a column. A magnetic field is applied so that the antibody-bound cells stick to the iron wool while cells that have not bound antibody are washed out; these cells are said to be negatively selected for lack of the molecule in question. The bound cells are released by removing the magnetic field; they are said to be positively selected for presence of the antigen recognized by the antibody.

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hybrids express the receptor of the normal T cell, but proliferate indefinitely owing to the cancerous state of the lymphoma parent. T-cell hybrids can be cloned to yield a population of cells all having the same T-cell receptor. When stimulated by their specific antigen, these cells release cytokines such as the T-cell growth factor interleukin-2 (IL-2), and the production of cytokines is used as an assay to assess the antigen specificity of the T-cell hybrid.

T-cell hybrids are excellent tools for the analysis of T-cell specificity, because they grow readily in suspension culture. However, they cannot be used to analyze the regulation of specific T-cell proliferation in response to antigen because they are continually dividing. T-cell hybrids also cannot be transferred into an animal to test for function *in vivo* because they would give rise to tumors. Functional analysis of T-cell hybrids is also confounded by the fact that the malignant partner cell affects their behavior in functional assays. Therefore, the regulation of T-cell growth and the effector functions of T cells must be studied using **T-cell clones**. These are clonal cell lines of a single T-cell type and antigen specificity, which are derived from cultures of heterogeneous T cells, called **T-cell lines**, whose growth is dependent on periodic restimulation with specific antigen and, frequently, on the addition of T-cell growth factors (Fig. A.28). T-cell clones also require periodic restimulation with antigen and are more tedious to grow than T-cell hybrids but, because their growth depends on specific antigen recognition, they maintain antigen specificity, which is often lost in T-cell hybrids. Cloned T-cell lines can be used for studies of effector function both *in vitro* and *in vivo*. In addition, the proliferation of T cells, a critical aspect of clonal selection, can be characterized only in cloned T-cell lines, where such growth is dependent on antigen recognition. Thus, both types of monoclonal T-cell lines have valuable applications in experimental studies.

Studies of human T cells have relied largely on T-cell clones because a suitable fusion partner for making T-cell hybrids has not been identified. However, a human T-cell lymphoma line, called Jurkat, has been characterized extensively because it secretes IL-2 when its antigen receptor is cross-linked with anti-receptor monoclonal antibodies. This simple assay system has yielded much information about signal transduction in T cells. One of the Jurkat cell line's most interesting features, shared with T-cell hybrids, is that it stops growing when its antigen receptor is cross-linked. This has allowed mutants lacking the receptor or having defects in signal transduction pathways to be selected simply by culturing the cells with anti-receptor antibody and selecting those that continue to grow. Thus, T-cell tumors, T-cell hybrids, and cloned T-cell lines all have valuable applications in experimental immunology.

Finally, primary T cells from any source can be isolated as single, antigen-specific cells by limiting dilution rather than by first establishing a mixed population of T cells in culture as a T-cell line and then deriving clonal subpopulations. During the growth of T-cell lines, particular T-cell clones can come to dominate the cultures and give a false picture of the number and specificities in the original sample. Direct cloning of primary T cells avoids this artifact.

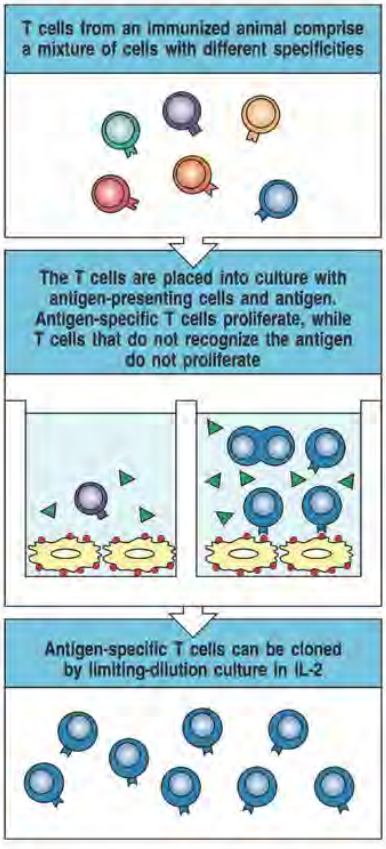


Fig. A.28 Production of cloned T-cell lines. T cells from an immune donor, comprising a mixture of cells with different specificities, are activated with antigen and antigen-presenting cells. Single responding cells are cultured by limiting dilution in the T-cell growth factor IL-2, which selectively stimulates the responding cells to proliferate. From these single cells, cloned lines specific for antigen are identified and can be propagated by culture with antigen, antigen-presenting cells, and IL-2.

Characterization of lymphocyte specificity, frequency, and function.

B cells are relatively easy to characterize because they have only one function—antibody production. T cells are more difficult to characterize because there are several different classes with different functions. It is also technically more difficult to study the membrane-bound T-cell receptors than

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the antibodies secreted in large amounts by B cells. All the methods in this part of the appendix can be used for T cells. Some are also used to detect and count B cells.

On many occasions it is important to know the frequency of antigen-specific lymphocytes, especially T cells, in order to measure the efficiency with which an individual responds to a particular antigen, for example, or the degree to which specific immunological memory has been established. There are a number of methods for doing this, either by detecting the cells directly by the specificity of their receptor, or by detecting activation of the cells to provide some particular function, such as cytokine secretion or cytotoxicity.

The first technique of this type to be established was the limiting-dilution culture (see Section A-25), in which the frequency of specific T or B cells responding to a particular antigen could be estimated by plating the cells into 96-well plates at increasing dilutions and measuring the number of wells in which there was no response. However, in this type of assay it became laborious to ask detailed questions about the phenotype of the responding cells, and to compare responses from different cell subpopulations.

A simpler assay for measuring the responses of T-cell populations has been developed from a variant of the antigen-capture ELISA method (see Section A-6), called the ELISPOT assay (see Section A-26). It assays T cells on the basis of cytokine production. In the ELISPOT assay, cytokine secreted by individual activated T cells is immobilized as discrete spots on a plastic plate, which are counted to give the number of activated T cells. The ELISPOT assay suffers from many of the same problems as the limiting-dilution assay in giving information about the nature of the activated cells, and it can be difficult to determine whether individual cells are capable of secreting mixtures of cytokines. It was therefore important to develop assays that could make these measurements on single cells. Measurements based on flow cytometry (see Section A-22) proved to be the answer, with the development of methods for detecting fluorescently labeled cytokines within activated T cells. The drawback of intracellular cytokine staining (see Section A-27) was that the T cells have to be killed and permeabilized by detergents to enable the cytokines to be detected. This led to the more sophisticated technique of capturing secreted labeled cytokines on the surfaces of the living T cells (see Section A-27).

Finally, methods for directly detecting T cells on the basis of the specificity of their receptor, using fluorochrome-tagged tetramers of specific peptide:MHC complexes (see Section A-28), have revolutionized the study of T-cell responses in a similar way to the use of monoclonal antibodies.

A-25 Limiting-dilution culture.

The response of a lymphocyte population is a measure of the overall response, but the frequency of lymphocytes able to respond to a given antigen can be determined only by **limiting-dilution culture**. This assay makes use of the Poisson distribution, a statistical function that describes how objects are distributed at random. For instance, when a sample of heterogeneous T cells is distributed equally into a series of culture wells, some wells will receive no T cells specific for a given antigen, some will receive one specific T cell, some two, and so on. The T cells in the wells are activated with specific antigen, antigen-presenting cells, and growth factors. After allowing several days for their growth and differentiation, the cells in each well are tested for a response to antigen, such as cytokine release or the ability to kill specific target cells (Fig. A.29). The assay is replicated with different numbers of T cells in the samples. The logarithm of the proportion of wells in which there is no response is plotted against the number of cells initially added to each well. If cells of one type, typically antigen-specific T cells because of their

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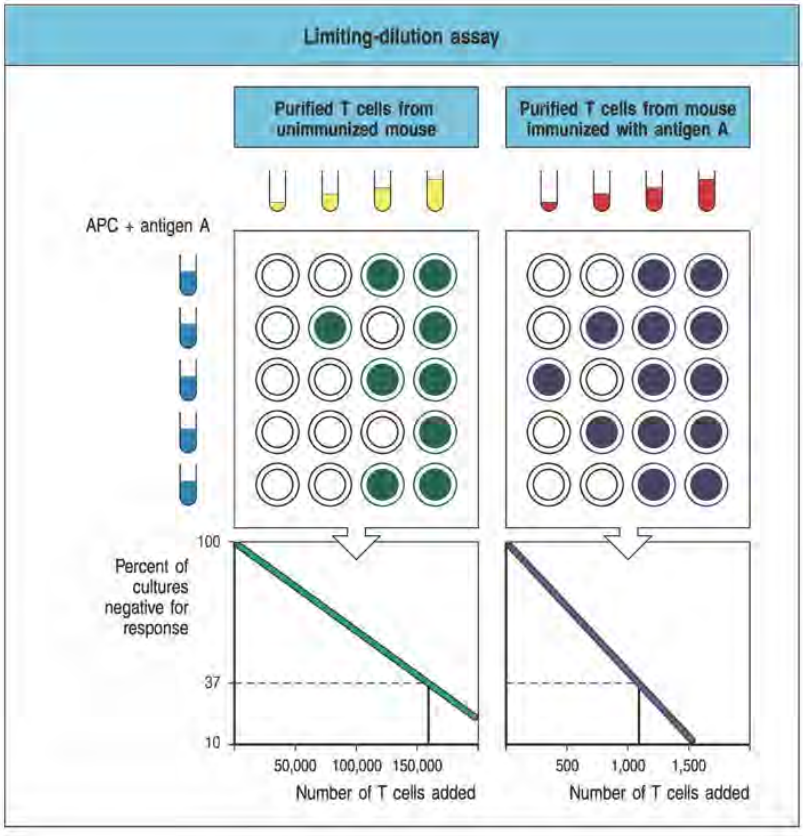


Fig. A.29 The frequency of specific lymphocytes can be determined using limiting-dilution assay. Various numbers of lymphoid cells from normal or immunized mice are added to individual culture wells and stimulated with antigen and antigen-presenting cells (APCs) or polyclonal mitogen and added growth factors. After several days, the wells are tested for a specific response to antigen, such as cytotoxic killing of target cells. Each well that initially contained a specific T cell will make a response to its target, and from the Poisson distribution one can determine that when 37% of the wells are negative, each well contained, on average, one specific T cell at the beginning of the culture. In the example shown, for the unimmunized mouse 37% of the wells are negative when 160,000 T cells have been added to each well; thus the frequency of antigen-specific T cells is 1 in 160,000. When the mouse is immunized, 37% of the wells are negative when only 1100 T cells have been added; hence the frequency of specific T cells after immunization is 1 in 1100, an increase in responsive cells of 150-fold.

rarity, are the only limiting factor for obtaining a response, then a straight line is obtained. From the Poisson distribution, it is known that there is, on average, one antigen-specific cell per well when the proportion of negative wells is 37%. Thus, the frequency of antigen-specific cells in the population equals the reciprocal of the number of cells added to each well when 37% of the wells are negative. After priming, the frequency of specific cells goes up substantially, reflecting the antigen-driven proliferation of antigen-specific cells. The limiting-dilution assay can also be used to measure the frequency of B cells that can make antibody against a given antigen.

A-26 ELISPOT assays.

A modification of the ELISA antigen-capture assay (see Section A-6), called the **ELISPOT assay**, has provided a powerful tool for measuring the frequency of T-cell responses. Populations of T cells are stimulated with the antigen of interest, and are then allowed to settle onto a plastic plate coated with antibodies against the cytokine that is to be assayed (Fig. A.30). If an activated T cell is secreting that cytokine, it is captured by the antibody on the plastic plate. After a period the cells are removed, and a second antibody against the cytokine is added to the plate to reveal a circle of bound cytokine surrounding the position of each activated T cell; counting each spot, and knowing the number of T cells originally added to the plate allows a simple calculation of the frequency of T cells secreting that particular cytokine, giving the ELISPOT assay its name. ELISPOT can also be used to detect specific antibody secretion by B cells, in this case by using antigen-coated surfaces to trap specific antibody and labeled anti-immunoglobulin to detect the bound antibody.

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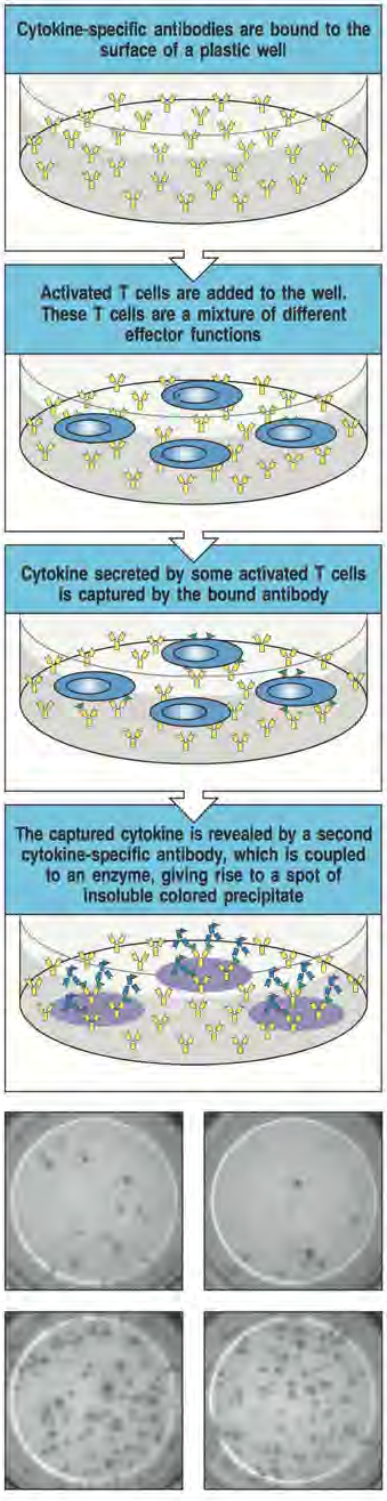


Fig. A.30 The frequency of cytokine-secreting T cells can be determined by the ELISPOT assay. The ELISPOT assay is a variant of the ELISA assay in which antibodies bound to a plastic surface are used to capture cytokines secreted by individual T cells. Usually, cytokine-specific antibodies are bound to the surface of a plastic tissue-culture well and the unbound antibodies are removed (top panel). Activated T cells are then added to the well and settle onto the antibody-coated surface (second panel). If a T cell is secreting the appropriate cytokine, this will then be captured by the antibody molecules on the plate surrounding the T cell (third panel). After a period of time the T cells are removed, and the presence of the specific cytokine is detected using an enzyme-labeled second antibody specific for the same cytokine. Where this binds, a colored reaction product can be formed (fourth panel). Each T cell that originally secreted cytokine gives rise to a single spot of color, hence the name of the assay. The results of such an ELISPOT assay for T cells secreting IFN-γ in response to different stimuli are shown in the last panel. In this example, T cells from a stem-cell transplant recipient were treated with a control peptide (top two panels) or a peptide from cytomegalovirus (bottom two panels). You can see a greater number of spots in the bottom two panels, indicating clearly that the patient's T cells are able to respond to the viral peptide and produce IFN-γ. Photographs courtesy of S. Nowack.

A-27 Identification of functional subsets of T cells by staining for cytokines.

One problem with the detection of cytokine production on a single-cell level is that the cytokines are secreted by the T cells into the surrounding medium, and any association with the originating cell is lost. Two methods have been devised that allow the cytokine profile produced by individual cells to be determined. The first, that of **intracellular cytokine staining** (Fig. A.31), relies on the use of metabolic poisons that inhibit protein export from the cell. The cytokine thus accumulates within the endoplasmic reticulum and vesicular network of the cell. If the cells are subsequently fixed and rendered permeable by the use of mild detergents, antibodies can gain access to these intracellular compartments and detect the cytokine. The T cells can be stained for other markers simultaneously, and thus the frequency of IL-10-producing $CD25^+$ CD4 T cells, for example, can be easily obtained.

A second method, which has the advantage that the cells being analyzed are not killed in the process, is called **cytokine capture**. This technique uses hybrid antibodies, in which the two separate heavy- and light-chain pairs from different antibodies are combined to give a mixed antibody molecule in which the two antigen-binding sites recognize different ligands (Fig. A.32). In the bispecific antibodies used to detect cytokine production, one of the antigen-binding sites is specific for a T-cell surface marker, while the other is specific for the cytokine in question. The bispecific antibody binds to the T cells through the binding site for the cell-surface marker, leaving the cytokine-binding site free. If that T cell is secreting the particular cytokine, it is captured by the bound antibody before it diffuses away from the surface of the cell. It can then be detected by adding a fluorochrome-labeled second antibody specific for the cytokine to the cells.

A-28 Identification of T-cell receptor specificity using peptide:MHC tetramers.

For many years, the ability to identify antigen-specific T cells directly through their receptor specificity eluded immunologists. Foreign antigen could not be used directly to identify T cells because, unlike B cells, they do not recognize antigen alone but rather the complexes of peptide fragments of antigen bound to self MHC molecules. Moreover, the affinity of interaction between the T-cell receptor and the peptide:MHC complex was in practice so low that attempts to label T cells with their specific peptide:MHC complexes routinely failed. The breakthrough in labeling antigen-specific T cells came with the idea of making multimers of the peptide:MHC complex, so as to increase the avidity of the interaction.

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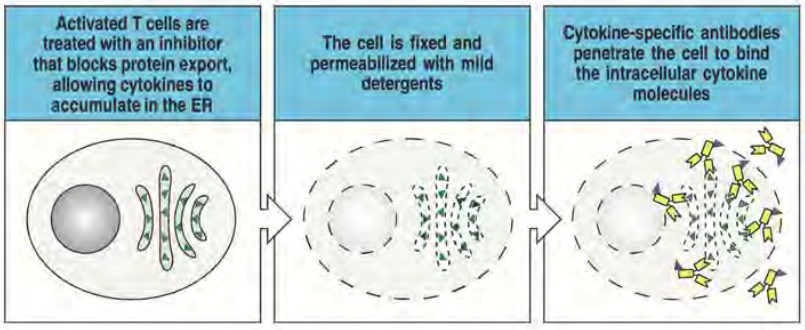


Fig. A.31 Cytokine-secreting cells can be identified by intracellular cytokine staining. The cytokines secreted by activated T cells can be determined by using fluorochrome-labeled antibodies to detect cytokine molecules that have been allowed to accumulate inside the cell. The accumulation of cytokine molecules, to allow them to reach a high enough concentration for efficient detection, is achieved by treating the activated T cells with inhibitors of protein export. In such treated cells, proteins destined to be secreted are instead retained within the endoplasmic reticulum (left panel). These treated cells are then fixed, to cross-link the proteins inside the cell and in the cell membranes, so that they are not lost when the cell is permeabilized by dissolving the cell membrane in a mild detergent (center panel). Fluorochrome-labeled antibodies can now enter the permeabilized cell and bind to the cytokines inside the cell (right panel). Cells labeled in this way can also be labeled with antibodies that bind to cell-surface proteins to determine which subsets of T cells are secreting particular cytokines.

Peptides can be biotinylated using the bacterial enzyme BirA, which recognizes a specific amino acid sequence. Recombinant MHC molecules containing this target sequence are used to make peptide:MHC complexes, which are then biotinylated. Avidin, or its bacterial counterpart streptavidin, contains four sites that bind biotin with extremely high affinity. Mixing the biotinylated peptide:MHC complex with avidin or streptavidin results in the formation of a **peptide:MHC tetramer**—four specific peptide:MHC complexes bound to a single molecule of streptavidin (Fig. A.33). Routinely, the streptavidin moiety is labeled with a fluorochrome to allow detection of those T cells capable of binding the peptide:MHC tetramer.

Peptide:MHC tetramers have been used to identify populations of antigen-specific T cells in, for example, patients with acute Epstein–Barr virus infections (infectious mononucleosis), showing that up to 80% of the peripheral T cells in infected individuals can be specific for a single peptide:MHC complex. They have also been used to follow responses over longer timescales in individuals with HIV or, in the example we show, cytomegalovirus infections. These reagents have also been important in identifying the cells responding, for example, to nonclassical class I molecules such as HLA-E or HLA-G, in

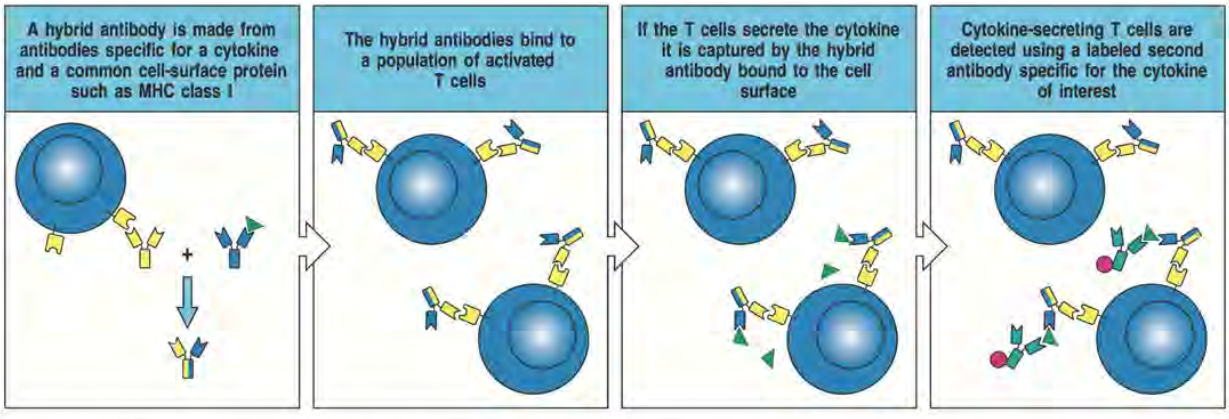


Fig. A.32 Hybrid antibodies containing cell-specific and cytokine-specific binding sites can be used to assay cytokine secretion by living cells and to purify cells secreting particular cytokines. Hybrid antibodies can be made by mixing together heavy- and light-chain pairs from antibodies of different specificities, for example an antibody against an MHC class I molecule and an antibody specific for a cytokine such as IL-4 (first panel). The hybrid antibodies are then added to a population of activated T cells, and bind to each cell via the MHC class I binding arm (second panel). If some of the cells in the population are secreting the appropriate cytokine, IL-4, this is captured by the cytokine-specific arm of the hybrid antibody (third panel). The presence of the cytokine can then be revealed, for example by using a fluorochrome-labeled second antibody specific for the same cytokine but binding to a different site to the one used for the hybrid antibody (last panel). The labeled cells are analyzed by flow cytometry or are isolated using a fluorescence-activated cell sorter. Alternatively, the second cytokine-specific antibody can be coupled to magnetic beads, and the cytokine-producing cells isolated magnetically.

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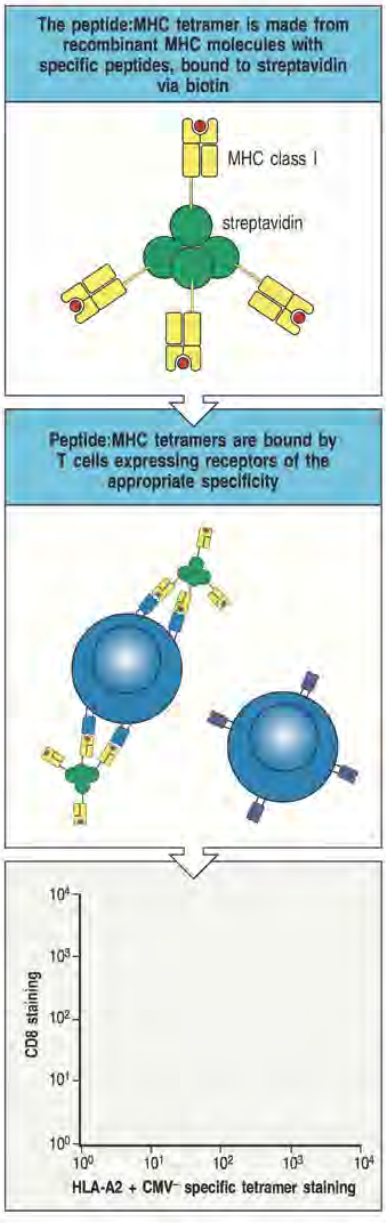


Fig. A.33 Peptide:MHC complexes coupled to streptavidin to form tetramers are able to stain antigen-specific T cells. Peptide:MHC tetramers are formed from recombinant refolded peptide:MHC complexes containing a single defined peptide epitope. The MHC molecules can be chemically derivatized to contain biotin, but more usually the recombinant MHC heavy chain is linked to a bacterial biotinylation sequence, a target for the *Escherichia coli* enzyme BirA, which is used to add a single biotin group to the MHC molecule. Streptavidin is a tetramer, each subunit having a single binding site for biotin; hence the streptavidin:peptide:MHC complex creates a tetramer of peptide:MHC complexes (top panel). Although the affinity between the T-cell receptor and its peptide:MHC ligand is too low for a single complex to bind stably to a T cell, the tetramer, by being able to make a more avid interaction with multiple peptide:MHC complexes binding simultaneously, is able to bind to T cells whose receptors are specific for the particular peptide:MHC complex (middle panel). Routinely, the streptavidin molecules are coupled to a fluorochrome, so that the binding to T cells can be monitored by flow cytometry. In the example shown in the bottom panel, T cells have been stained simultaneously with antibodies specific for CD3 and CD8, and with a tetramer of HLA-A2 molecules containing a cytomegalovirus peptide. Only the $CD3^+$ cells are shown, with the staining of CD8 displayed on the vertical axis and the tetramer staining displayed along the horizontal axis. The $CD8^-$ cells (mostly $CD4^+$) on the bottom left of the figure show no specific tetramer staining, while the bulk of the $CD8^+$ cells, on the top left, likewise show no tetramer staining. However, a discrete population of tetramer positive $CD8^+$ cells, at the top right of the panel, comprising some 5% of the total $CD8^+$ cells, can clearly be seen. Data courtesy of G. Aubert.

both cases showing that these nonclassical molecules are recognized by subsets of NK receptors.

A-29 Assessing the diversity of the T-cell repertoire by 'spectratyping.'

The extent of the diversity of the T-cell repertoire, either generally or during specific immune responses, is often of interest. In particular, as T cells do not undergo somatic hypermutation and affinity maturation in the same way that B cells do, the relationship between the repertoire of T cells making a primary response to antigen and the repertoire of T cells involved in secondary and subsequent responses to antigen has been difficult to determine. This information has usually been obtained through the laborious process of cloning the T cells involved in specific responses (see Section A-24), and the cloning and sequencing of their T-cell receptors.

It is possible, however, to estimate the diversity of T-cell responses by making use of the junctional diversity generated when T-cell receptors are created by somatic recombination, a technique known as **spectratyping**. Variability in the length of the CDR3 segments is created during the recombination process, both by variation in the exact positions at which the junctions between gene segments occur and by variation in the number of N-nucleotides added. Both these processes result in the length of the V_β CDR3 varying by up to nine amino acids. The problem in detecting this variability is that there are 24 families of V_β gene segments in humans and it is not possible to design a single oligonucleotide primer that will anneal to all of these families. Specific oligonucleotide primers can, however, be designed for each family, and these can be used in the polymerase chain reaction (PCR), together with a primer specific for the C_β region, to amplify, for each individual family, a segment of the mRNA for the T-cell receptor β chain that spans the CDR3 region. A population of TCR V_β genes will therefore show a distribution, or 'spectrum,' of CD3 lengths, and will give rise to PCR products of different lengths that can be resolved, usually by polyacrylamide gel electrophoresis (Fig. A.34). The deletion and addition of nucleotides during the generation of T-cell receptors by rearrangement is random, and so in a normal individual the CDR3 lengths follow a Gaussian distribution. Deviations from this Gaussian distribution, such as an excess of one particular CDR3 length, indicate the presence of clonal expansions of T cells, such as occurs during a T-cell response.

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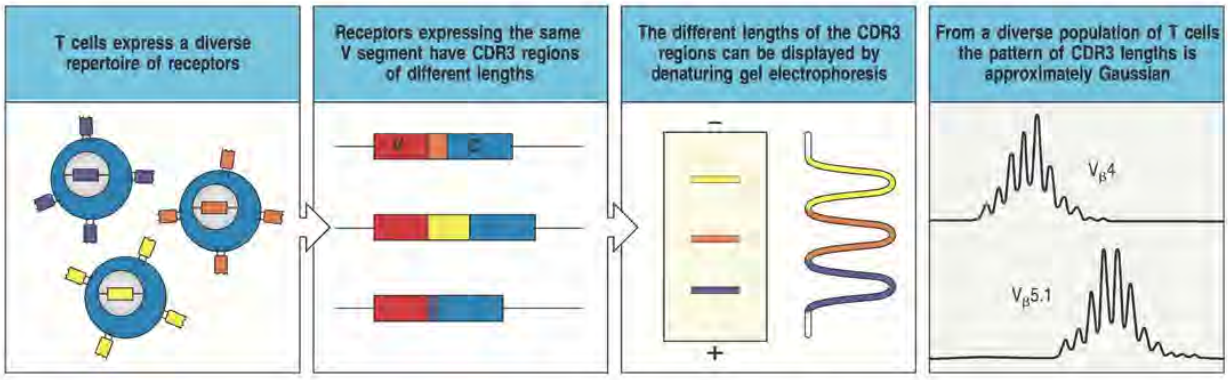


Fig. A.34 The diversity of the T-cell receptor repertoire can be displayed by spectratyping, a PCR-based technique that separates different receptors on the basis of their CDR3 length. The process of generation of T-cell receptors is stochastic, giving rise to a population of mature T cells whose receptors are clonally distributed (first panel). In each of the cells expressing a particular V_β gene segment, all of the differences between the unique receptors are restricted to the CDR3 region, where there will be differences in length as well as sequence as a consequence of the imprecision of the rearrangement process (second panel). Using sets of primers for the PCR reaction that are specific for individual V_β gene segments at one end and for a conserved part of the C region at the other, it is possible to generate a set of DNA fragments that span the CDR3 region. If these are separated by denaturing acrylamide gel electrophoresis, a series of bands are formed or, because these fragments can be labeled with fluorochromes and analyzed by automated gel readers, a series of peaks corresponding to the different length fragments (third panel). The pattern of peaks obtained in this way is known as a spectratype. From a diverse population of cells, the distribution of fragment lengths is Gaussian, as shown in the last panel, in which the spectratypes of two different V_β regions from the same individual are shown. In this case, both of the patterns are approximately Gaussian; deviations from a Gaussian distribution may indicate expansion of particular clones of T cells, perhaps in response to antigenic challenge. Data courtesy of L. McGreavey.

A-30 Biosensor assays for measuring the rates of association and disassociation of antigen receptors for their ligands.

Two of the important questions that are always asked of any receptor–ligand interaction are: what is the strength of binding, or affinity, of the interaction, and what are the rates of association and disassociation? Traditionally, measurements of affinity were made by equilibrium binding measurements (see Section A-9), and measurements of rates of binding were difficult to obtain. In addition, equilibrium binding assays cannot be performed on T-cell receptors, which have large macromolecular ligands and cannot be isolated and purified in large quantity.

It is now possible to measure binding rates directly, by following the binding of ligands to receptors immobilized on gold-plated glass slides, using a phenomenon known as **surface plasmon resonance** (**SPR**) to detect the binding (Fig. A.35). A full explanation of surface plasmon resonance is beyond the scope of this textbook, as it is based on advanced physical and quantum mechanical principles. In brief, it relies on the total internal reflection of a beam of light from the surface of a gold-coated glass slide. As the light is reflected, some of its energy excites electrons in the gold coating and these excited electrons are in turn affected by the electric field of any molecules binding to the surface of the glass coating. The more molecules that bind to the surface, the greater is the effect on the excited electrons, and this in turn affects the reflected light beam. The reflected light thus becomes a sensitive measure of the number of atoms bound to the gold surface of the slide.

If a purified receptor is immobilized on the surface of the gold-coated glass slide, to make a biosensor 'chip,' and a solution containing the ligand is flowed over that surface, the binding of ligand to the receptor can be followed until it reaches equilibrium (see Fig. A.35). If the ligand is then washed out, dissociation of ligand from the receptor can easily be followed and the dissociation rate

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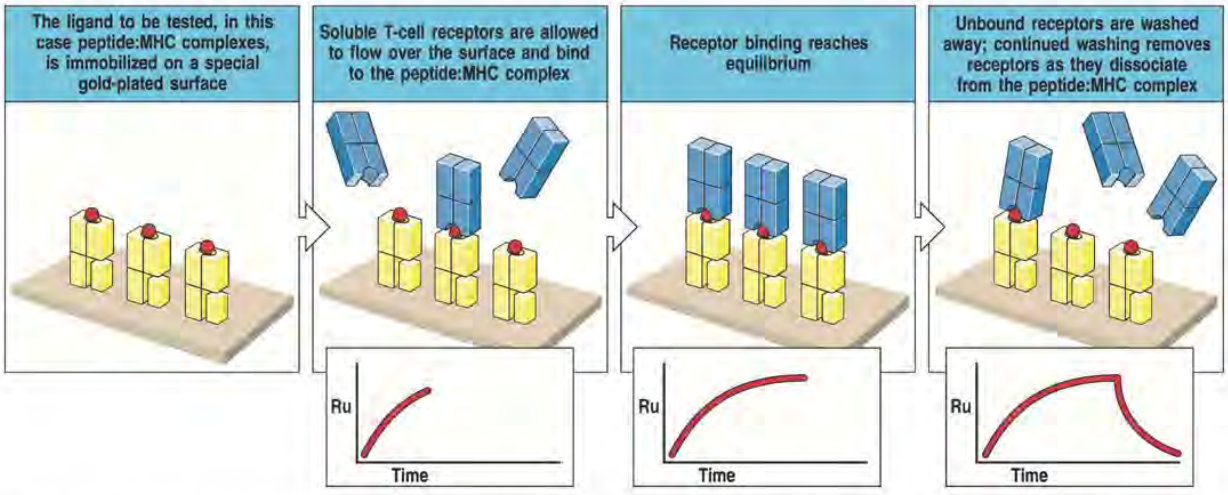


Fig. A.35 Measurement of receptor–ligand interactions can be made in real time using a biosensor. Biosensors are able to measure the binding of molecules on the surface of gold-plated glass chips through the indirect effects of the binding on the total internal reflection of a beam of polarized light at the surface of the chip. Changes in the angle and intensity of the reflected beam are measured in 'resonance units' (Ru) and plotted against time in what is termed a 'sensorgram.' Depending on the exact nature of the receptor–ligand pair to be analyzed, either the receptor or the ligand can be immobilized on the surface of the chip. In the example shown, peptide:MHC complexes are immobilized on such a surface (first panel). T-cell receptors in solution are now allowed to flow over the surface, and to bind to the immobilized peptide:MHC complexes (second panel). As the T-cell receptors bind, the sensorgram (inset panel below the main panel) reflects the increasing amount of protein bound. As the binding reaches either saturation or equilibrium (third panel), the sensorgram shows a plateau, as no more protein binds. At this point, unbound receptors can be washed away. With continued washing, bound receptors now start to dissociate and are removed in the flow of the washing solution (last panel). The sensorgram now shows a declining curve, reflecting the rate at which the receptor and ligand dissociation occurs.

calculated. A new solution of the ligand at a different concentration can then be flowed over the chip and the binding once again measured. The affinity of binding can be calculated in a number of ways in this type of assay. Most simply, the ratio of the rates of association and dissociation will give an estimate of the affinity, but more accurate estimates can be obtained from the measurements of the binding at different concentrations of ligand. From measurements of binding at equilibrium, a Scatchard plot (see Fig. A.11) will give a measurement of the affinity of the receptor–ligand interaction.

Mitogen	Responding cells
Phytohemagglutinin (PHA) (red kidney bean)	T cells
Concanavalin (ConA) (jack bean)	T cells
Pokeweed mitogen (PWM) (pokeweed)	T and B cells
Lipopolysaccharide (LPS) (*Escherichia coli*)	B cells (mouse)

Fig. A.36 Polyclonal mitogens, many of plant origin, stimulate lymphocyte proliferation in tissue culture. Many of these mitogens are used to test the ability of lymphocytes in human peripheral blood to proliferate.

A-31 Stimulation of lymphocyte proliferation by treatment with polyclonal mitogens or specific antigen.

To function in adaptive immunity, rare antigen-specific lymphocytes must proliferate extensively before they differentiate into functional effector cells in order to generate sufficient numbers of effector cells of a particular specificity. Thus, the analysis of induced lymphocyte proliferation is a central issue in their study. It is, however, difficult to detect the proliferation of normal lymphocytes in response to specific antigen because only a minute proportion of cells will be stimulated to divide. Great impetus was given to the field of lymphocyte culture by the finding that certain substances induce many or all lymphocytes of a given type to proliferate. These substances are referred to collectively as **polyclonal mitogens** because they induce mitosis in lymphocytes of many different specificities or clonal origins. T and B lymphocytes are stimulated by different polyclonal mitogens (Fig. A.36). Polyclonal mitogens seem to trigger essentially the same growth response mechanisms

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as antigen. Lymphocytes normally exist as resting cells in the G_0 phase of the cell cycle. When stimulated with polyclonal mitogens, they rapidly enter the G_1 phase and progress through the cell cycle. In most studies, lymphocyte proliferation is most simply measured by the incorporation of ^{3}H-thymidine into DNA. This assay is used clinically for assessing the ability of lymphocytes from patients with suspected immunodeficiencies to proliferate in response to a nonspecific stimulus.

Once lymphocyte culture had been optimized using the proliferative response to polyclonal mitogens as an assay, it became possible to detect antigen-specific T-cell proliferation in culture by measuring ^{3}H-thymidine uptake in response to an antigen to which the T-cell donor had previously been immunized (Fig. A.37). This is the assay most commonly used for assessing T-cell responses after immunization, but it reveals little about the functional capabilities of the responding T cells. These must be ascertained by functional assays, as outlined in Sections A-33 and A-34.

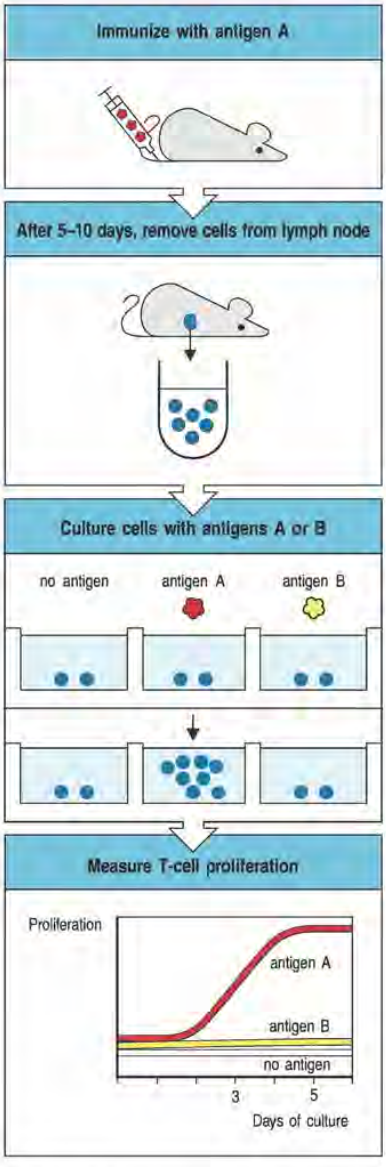


Fig. A.37 Antigen-specific T-cell proliferation is used frequently as an assay for T-cell responses. T cells from mice or humans that have been immunized with an antigen (A) proliferate when they are exposed to antigen A and antigen-presenting cells but not when cultured with unrelated antigens to which they have not been immunized (antigen B). Proliferation can be measured by incorporation of ^{3}H-thymidine into the DNA of actively dividing cells. Antigen-specific proliferation is a hallmark of specific CD4 T-cell immunity.

A-32 Measurements of apoptosis by the TUNEL assay.

Apoptotic cells can be detected by a procedure known as **TUNEL staining**. In this technique, the 3′ ends of the DNA fragments generated in apoptotic cells are labeled with biotin-coupled uridine by using the enzyme terminal deoxynucleotidyl transferase (TdT). The biotin label is then detected with enzyme-tagged streptavidin, which binds to biotin. When the colorless substrate of the enzyme is added to a tissue section or cell culture, it is reacted on to produce a colored precipitate only in cells that have undergone apoptosis (Fig. A.38). This technique has revolutionized the detection of apoptotic cells.

A-33 Assays for cytotoxic T cells.

Activated CD8 T cells generally kill any cells that display the specific peptide:MHC class I complex they recognize. Thus, CD8 T-cell function can be determined using the simplest and most rapid T-cell bioassay—the killing of a target cell by a cytotoxic T cell. This is usually detected in a ^{51}Cr-release assay. Live cells will take up, but do not spontaneously release, radioactively labeled sodium chromate, $Na_2{}^{51}CrO_4$. When these labeled cells are killed, the radioactive chromate is released and its presence in the supernatant of mixtures of target cells and cytotoxic T cells can be measured (Fig. A.39). In a similar assay, proliferating target cells such as tumor cells can be labeled with ^{3}H-thymidine, which is incorporated into the replicating DNA. On attack by a cytotoxic T cell, the DNA of the target cells is rapidly fragmented and retained in the filtrate, while large, unfragmented DNA is collected on a filter, and one can measure either the release of these fragments or the retention of ^{3}H-thymidine in chromosomal DNA. These assays provide a rapid, sensitive, and specific measure of the activity of cytotoxic T cells.

A-34 Assays for CD4 T cells.

CD4 T-cell functions usually involve the activation rather than the killing of cells bearing specific antigen. The activating effects of CD4 T cells on B cells or macrophages are mediated in large part by nonspecific mediator proteins called cytokines, which are released by the T cell when it recognizes antigen. Thus, CD4 T-cell function is usually studied by measuring the type and amount of these released proteins. Because different effector T cells release different amounts and types of cytokines, one can learn about the effector potential of that T cell by measuring the proteins it produces.

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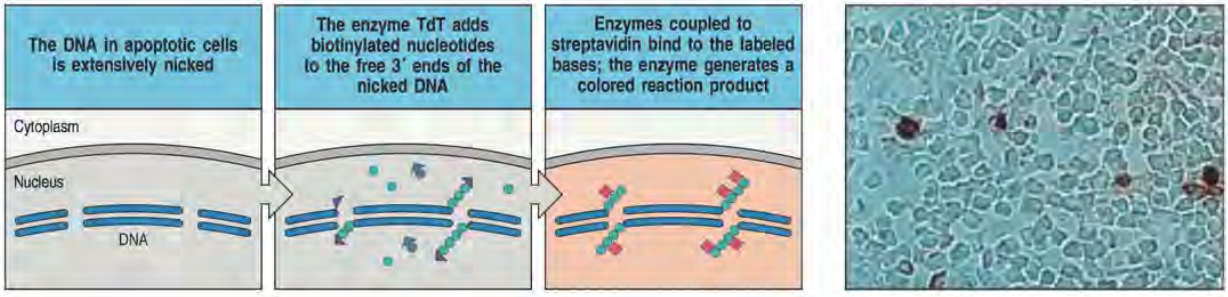


Fig. A.38 In the TUNEL assay, fragmented DNA is labeled by terminal deoxynucleotidyl transferase (TdT) to reveal apoptotic cells. When cells undergo programmed cell death, or apoptosis, their DNA becomes fragmented (first panel). The enzyme TdT is able to add nucleotides to the ends of DNA fragments; most commonly in this assay, biotin-labeled nucleotides (usually dUTP) are added (second panel). The biotinylated DNA can be detected by using streptavidin, which binds to biotin, coupled to enzymes that convert a colorless substrate into a colored insoluble product (third panel). Cells stained in this way can be detected by light microscopy, as shown in the photograph of apoptotic cells (stained red) in the thymic cortex. Photograph courtesy of R. Budd and J. Russell.

Cytokines can be detected by their activity in biological assays of cell growth, where they serve either as growth factors or as growth inhibitors. A more specific assay is a modification of ELISA known as a capture or sandwich ELISA (see Section A-6). In this assay, the cytokine is characterized by its ability to act as a bridge between two monoclonal antibodies reacting with different epitopes on the cytokine molecule. Cytokine-secreting cells can also be detected by ELISPOT (see Section A-26).

Sandwich ELISA and ELISPOT avoid a major problem of cytokine bioassays, namely the ability of different cytokines to stimulate the same response in a bioassay. Bioassays must always be confirmed by inhibition of the response with neutralizing monoclonal antibodies specific for the cytokine. Another way of identifying cells actively producing a given cytokine is to stain them with a fluorescently tagged anti-cytokine monoclonal antibody, and then identify and count them by FACS (see Section A-22).

A quite different approach to detecting cytokine production is to determine the presence and amount of the relevant cytokine mRNA in stimulated T cells. This can be done for single cells by *in situ* hybridization and for cell populations by **reverse transcriptase–polymerase chain reaction (RT-PCR)**. Reverse transcriptase is an enzyme used by certain RNA viruses, such as HIV, to convert an RNA genome into a DNA copy, or cDNA. In RT–PCR, mRNA is isolated from cells and cDNA copies are made *in vitro* using reverse transcriptase. The desired cDNA is then selectively amplified by PCR by using sequence-specific primers. When the products of the reaction are subjected to electrophoresis on an agarose gel, the amplified DNA can be visualized as a band of a specific size. The amount of amplified cDNA sequence will be proportional to its representation in the mRNA; stimulated T cells actively producing a particular cytokine will produce large amounts of that particular mRNA and will thus give correspondingly large amounts of the selected cDNA on RT–PCR. The level of cytokine mRNA in the original tissue is usually determined by comparison with the outcome of RT–PCR on the mRNA produced by a so-called 'housekeeping gene' expressed by all cells.

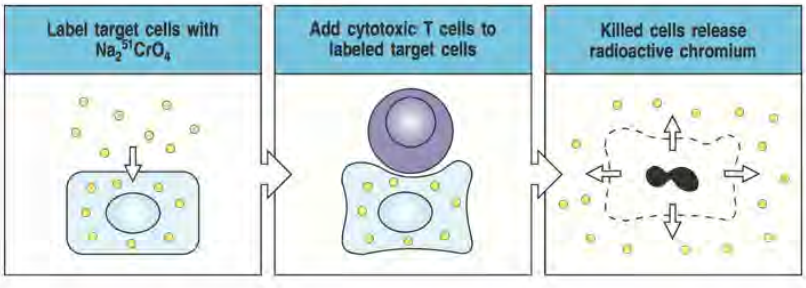


Fig. A.39 Cytotoxic T-cell activity is often assessed by chromium release from labeled target cells. Target cells are labeled with radioactive chromium as $Na_2{}^{51}CrO_4$, washed to remove excess radioactivity, and exposed to cytotoxic T cells. Cell destruction is measured by the release of radioactive chromium into the medium, detectable within 4 hours of mixing target cells with T cells.

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Detection of immunity *in vivo*.

A-35 Assessment of protective immunity.

An adaptive immune response against a pathogen often confers long-lasting immunity against infection with that pathogen; successful vaccination achieves the same end. The very first experiment in immunology, Jenner's successful vaccination against smallpox, is still the model for assessing the presence of such protective immunity. The assessment of protective immunity conferred by vaccination has three essential steps. First, an immune response is elicited by immunization with a candidate vaccine. Second, the immunized individuals, along with unimmunized controls, are challenged with the infectious agent (Fig. A.40). Finally, the prevalence and severity of infection in the immunized individual are compared with the course of the disease in the unimmunized controls. For obvious reasons, such experiments are usually performed first in animals, if a suitable animal model for the infection exists. However, eventually a trial must be conducted in humans. In this case, the infectious challenge is usually provided naturally by conducting the trial in a region where the disease is prevalent. The efficacy of the vaccine is determined by assessing the prevalence and severity of new infections in the immunized and control populations. Such studies necessarily give less precise results than a direct experiment; however, for most diseases they are the only way of assessing a vaccine's ability to induce protective immunity in humans.

A-36 Transfer of protective immunity.

The tests described in Section A-35 show that protective immunity has been established, but cannot show whether it involves humoral immunity, cell-mediated immunity, or both. When these studies are performed in inbred mice, the nature of protective immunity can be determined by transferring serum or lymphoid cells from an immunized donor animal to an unimmunized syngeneic recipient (that is, a genetically identical animal of the same inbred strain) (Fig. A.41). If protection against infection can be conferred by the transfer of serum, the immunity is provided by circulating antibodies and is called **humoral immunity**. Transfer of immunity by antiserum or purified antibodies provides immediate protection against many pathogens and against toxins such as those of tetanus and snake venom. However, although protection is immediate, it is temporary, lasting only so long as the transferred antibodies remain active in the recipient's body. This type of transfer is therefore called **passive immunization**. Only **active immunization** with antigen can provide lasting immunity. Moreover, the recipient may become immunized to the antiserum used to transfer immunity. Horse or sheep sera are the usual sources of anti-snake venoms used in humans, and repeated administration can lead either to serum sickness (see Section 14-16) or, if the recipient becomes allergic to the foreign serum, to anaphylaxis (see Section 14-10).

Protection against many diseases cannot be transferred with serum but can be transferred by lymphoid cells from immunized donors. The transfer of lymphoid cells from an immune donor to a normal syngeneic recipient is called **adoptive transfer** or **adoptive immunization**, and the immunity transferred is called **adoptive immunity**. Immunity that can be transferred only with lymphoid cells is called **cell-mediated immunity**. Such cell transfers must be between genetically identical donors and recipients, such as members of the same inbred strain of mouse, so that the donor lymphocytes are not rejected by the recipient and do not attack the recipient's tissues. Adoptive transfer of immunity is used clinically in humans in experimental approaches to cancer

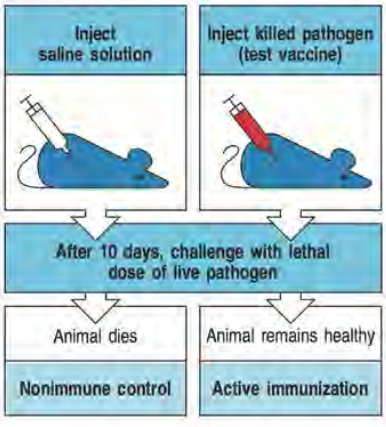


Fig. A.40 *In vivo* assay for the presence of protective immunity after vaccination in animals. Mice are injected with the test vaccine or a control such as saline solution. Different groups are then challenged with lethal or pathogenic doses of the test pathogen or with an unrelated pathogen as a specificity control (not shown). Unimmunized animals die or become severely infected. Successful vaccination is seen as specific protection of immunized mice against infection with the test pathogen. This is called active immunity, and the process is called active immunization.

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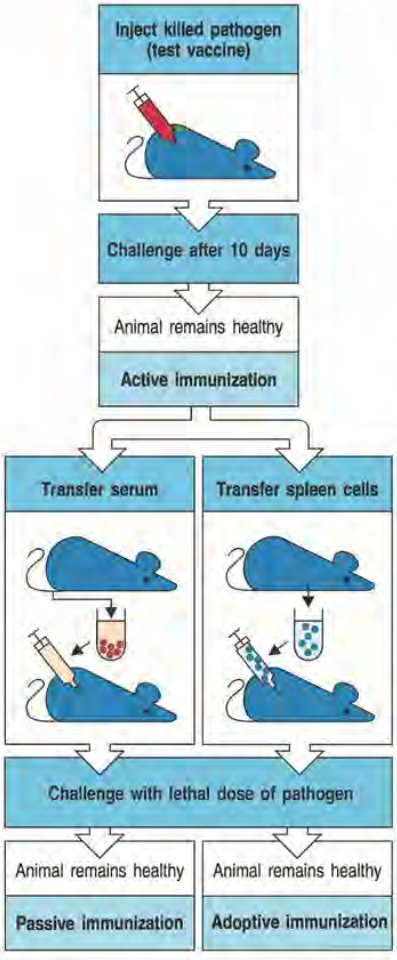


Fig. A.41 Immunity can be transferred by antibodies or by lymphocytes. Successful vaccination leads to a long-lived state of protection against the specific immunizing pathogen. If this immune protection can be transferred to a normal syngeneic recipient with serum from an immune donor, then immunity is mediated by antibodies; such immunity is called humoral immunity and the process is called passive immunization. If immunity can be transferred only by infusing lymphoid cells from the immune donor into a normal syngeneic recipient, then the immunity is called cell-mediated immunity and the transfer process is called adoptive transfer or adoptive immunization. Passive immunity is short-lived, because antibody is eventually catabolized, but adoptively transferred immunity is mediated by immune cells, which can survive and provide longer-lasting immunity.

therapy or as an adjunct to bone marrow transplantation; in these cases, the patient's own T cells, or the T cells of the bone marrow donor, are given.

A-37 The tuberculin test.

Local responses to antigen can indicate the presence of active immunity. Active immunity is often studied *in vivo*, especially in humans, by injecting antigens locally in the skin. If a reaction appears, this indicates the presence of antibodies or immune lymphocytes that are specific for that antigen; the **tuberculin test** is an example of this. When people have had tuberculosis they develop cell-mediated immunity that can be detected as a local response when their skin is injected with a small amount of tuberculin, an extract of *Mycobacterium tuberculosis*, the pathogen that causes tuberculosis. The response typically appears a day or two after the injection and consists of a raised, red, and hard (or indurated) area in the skin, which then disappears as the antigen is degraded.

A-38 Testing for allergic responses.

Local intracutaneous injections of minute doses of the antigens that cause allergies are used to determine which antigen triggers a patient's allergic reactions. Local responses that happen in the first few minutes after antigen injection in immune recipients are called **immediate hypersensitivity reactions**, and they can be of several forms, one of which is the wheal-and-flare response (see Fig. 14.13). Immediate hypersensitivity reactions are mediated by specific antibodies of the IgE class formed as a result of earlier exposures to the antigen. Responses that take hours to days to develop, such as the tuberculin test, are referred to as **delayed-type hypersensitivity responses** and are caused by preexisting immune T cells. This latter type of response was observed by Jenner when he tested vaccinated individuals with a local injection of vaccinia virus.

These tests work because the local deposit of antigen remains concentrated in the initial site of injection, eliciting responses in local tissues. They do not cause generalized reactions if sufficiently small doses of antigen are used. However, local tests carry a risk of systemic allergic reactions, and they should be used with caution in people with a history of hypersensitivity.

A-39 Assessment of immune responses and immunological competence in humans.

The methods used for testing immune function in humans are necessarily more limited than those used in experimental animals, but many different tests are available. They fall into several groups depending on the reason the patient is being studied.

Assessment of protective immunity in humans generally relies on tests conducted *in vitro*. To assess humoral immunity, specific antibody levels in the patient's serum are assayed by RIA or, more commonly, ELISA (see Section A-6), using the test microorganism or a purified microbial product as antigen. To test for humoral immunity against viruses, antibody production is often measured by the ability of serum to neutralize the infectivity of live virus for tissue culture cells. In addition to providing information about protective immunity, the presence of antibody against a particular pathogen indicates that the patient has been exposed to it, making such tests of crucial importance in epidemiology. At present, testing for antibody against HIV is the main screening test for infection with this virus, critical both for the patient and in blood banking, where blood from infected donors must be excluded from the supply. Essentially similar tests are used in investigating allergy, where

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allergens are used as the antigens in tests for specific IgE antibody by ELISA or RIA (see Section A-6), which may be used to confirm the results of skin tests.

Cell-mediated immunity—that is, immunity mediated by T cells—is technically more difficult to measure than humoral immunity. This is principally because T cells do not make a secreted antigen-binding product, so there is no simple binding assay for their antigen-specific responses. T-cell activity can be divided into an induction phase, in which T cells are activated to divide and differentiate, and an effector phase, in which their function is expressed. Both phases require that the T cell interact with another cell and that it recognize specific antigen displayed in the form of peptide:MHC complexes on the surface of this interacting cell. In the induction phase, the interaction must be with an antigen-presenting cell able to deliver co-stimulatory signals, whereas, in the effector phase, the nature of the target cell depends on the type of effector T cell that has been activated. Most commonly, the presence of T cells that have responded to a specific antigen is detected by their subsequent *in vitro* proliferation when reexposed to the same antigen (see Section A-31).

T-cell proliferation indicates only that cells able to recognize that antigen have been activated previously; it does not reveal what effector function they mediate. The effector function of a T cell is assayed by its effect on an appropriate target cell. Assays for cytotoxic CD8 T cells (see Section A-33) and for cytokine production by CD4 T cells (see Sections A-26, A-27, and A-34) are used to characterize the immune response. Cell-mediated immunity to infectious agents can also be tested by skin test with extracts of the pathogen, as in the tuberculin test (see Section A-37). These tests provide information about the exposure of the patient to the disease and also about the patient's ability to mount an adaptive immune response to it.

Patients with immune deficiency (see Chapter 13) are usually detected clinically by a history of recurrent infection. To determine the competence of the immune system in such patients, a battery of tests are usually conducted; these focus with increasing precision as the nature of the defect is narrowed down to a single element. The presence of the various cell types in blood is determined by routine hematology, often followed by FACS analysis of lymphocyte subsets (see Section A-22) and the measurement of serum immunoglobulins. Figure A.42 lists the main tests that are performed and the 'normal' ranges for leukocyte numbers and concentrations of each antibody class in peripheral blood. The phagocytic competence of freshly isolated polymorphonuclear leukocytes and monocytes is tested, and the efficiency of the complement system (see Chapters 2 and 9) is determined by testing the dilution of serum required for the lysis of 50% of antibody-coated red blood cells (this is denoted the CH_{50}).

In general, if such tests reveal a defect in one of the broad compartments of immune function, more specialized testing is then needed to determine the precise nature of the defect. Tests of lymphocyte function are often valuable, starting with the ability of polyclonal mitogens to induce T-cell proliferation and B-cell secretion of immunoglobulin in tissue culture (see Section A-31). These tests can eventually pinpoint the cellular defect in immunodeficiency.

In patients with autoimmune diseases (see Chapter 15), the same parameters are usually analyzed to determine whether there is a gross abnormality in the immune system. However, most patients with such diseases show few abnormalities in general immune function. To determine whether a patient is producing antibody against their own cellular antigens, the most informative test is to react their serum with tissue sections, which are then examined for bound antibody by indirect immunofluorescence using anti-human immunoglobulin labeled with fluorescent dye (see Section A-14). Most autoimmune diseases are associated with the production of broadly characteristic patterns of autoantibodies directed at self tissues. These patterns aid

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Fig. A.42 Evaluation of immune function.

	Evaluation of the cellular components of the human immune system		
	B cells	**T cells**	**Phagocytes**
Normal numbers ($\times 10^9$ per liter of blood)	Approximately 0.3	Total 1.0–2.5 CD4 0.5–1.6 CD8 0.3–0.9	Monocytes 0.15–0.6 Polymorphonuclear leukocytes Neutrophils 3.00–5.5 Eosinophils 0.05–0.25 Basophils 0.02
Measurement of function *in vivo*	Serum Ig levels Specific antibody levels	Skin test	—
Measurement of function *in vitro*	Induced antibody production in response to pokeweed mitogen	T-cell proliferation in response to phytohemagglutinin or to tetanus toxoid	Phagocytosis Nitro blue tetrazolium uptake Intracellular killing of bacteria

	Evaluation of the humoral components of the human immune system				
	Immunoglobulins				**Complement**
Component	IgG	IgM	IgA	IgE	
Normal levels	600–1400 $mg\,dl^{-1}$	40–345 $mg\,dl^{-1}$	60–380 $mg\,dl^{-1}$	0–200 $IU\,ml^{-1}$	CH_{50} of 125–300 $IU\,ml^{-1}$

in the diagnosis of the disease and help to distinguish autoimmunity from tissue inflammation due to infectious causes.

It is also possible to investigate allergies by administering possible allergens by routes other than intracutaneous administration. Allergen may be given by inhalation to test for asthmatic allergic responses (see Fig. 14.13); this is done mainly for experimental purposes in studies of the mechanisms and treatment of asthma. Similarly, food allergens may be given by mouth. The administration of allergens is potentially very dangerous because of the risk of causing anaphylaxis, and must only be done by trained and experienced investigators in an environment in which full resuscitation facilities are available.

A-40 The Arthus reaction.

This inflammatory skin reaction (see Section 14-16) can be induced in animal models to study the formation of immune complexes in tissues and how they cause inflammation. The original reaction described by Maurice Arthus was induced by the repeated injection of horse serum into rabbits. Initial injections of horse serum into the skin induced no reaction, but later injections, after the production of antibodies against the proteins in horse serum, induced an inflammatory reaction at the site of injection after several hours, characterized by the presence of edema, hemorrhage, and neutrophil infiltration, which frequently progressed to tissue necrosis. Most investigators now use passive models of the Arthus reaction in which either antibody is infused systemically and antigen is given locally (passive Arthus reaction) or antigen is infused systemically and antibody is injected locally (reverse passive Arthus reaction).

A-41 Adoptive transfer of lymphocytes.

Ionizing radiation from X-ray or γ-ray sources kills lymphoid cells at doses that spare the other tissues of the body. This makes it possible to eliminate

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immune function in a recipient animal before attempting to restore immune function by adoptive transfer, and allows the effect of the adoptively transferred cells to be studied in the absence of other lymphoid cells. James Gowans originally used this technique to prove the role of the lymphocyte in immune responses. He showed that all active immune responses could be transferred to irradiated recipients by small lymphocytes from immunized donors. This technique can be refined by transferring only certain lymphocyte subpopulations, such as B cells or CD4 T cells. Even cloned T-cell lines have been tested for their ability to transfer immune function, and have been shown to confer adoptive immunity to their specific antigen. Such adoptive transfer studies are a cornerstone in the study of the intact immune system, as they can be performed rapidly, simply, and in any strain of mouse.

A-42 Hematopoietic stem-cell transfers.

All cells of hematopoietic origin can be eliminated by treatment with high doses of X rays, allowing replacement of the entire hematopoietic system, including lymphocytes, by transfusion of donor bone marrow or purified hematopoietic stem cells from another animal. The resulting animals are called **radiation bone marrow chimeras** from the Greek word *chimera*, a mythical animal that had the head of a lion, the tail of a serpent, and the body of a goat. This technique is used experimentally to examine the development of lymphocytes, as opposed to their effector functions, and has been particularly important in studying T-cell development. Essentially the same technique is used in humans to replace the hematopoietic system when it fails, as in aplastic anemia or after nuclear accidents, or to eradicate the bone marrow and replace it with normal marrow in the treatment of certain cancers. In humans, bone marrow is the main source of hematopoietic stem cells, but they are increasingly being obtained from peripheral blood after the donor has been treated with hematopoietic growth factors such as GM-CSF, or from umbilical cord blood, which is rich in such stem cells (see Chapter 15).

A-43 *In vivo* depletion of T cells.

The importance of T-cell function *in vivo* can be ascertained in mice with no T cells of their own. Under these conditions, the effect of a lack of T cells can be studied, and T-cell subpopulations can be restored selectively to analyze their specialized functions. T lymphocytes originate in the thymus, and neonatal **thymectomy**, the surgical removal of the thymus of a mouse at birth, prevents T-cell development from occurring because the export of most functionally mature T cells occurs only after birth in the mouse. Alternatively, adult mice can be thymectomized and then irradiated and reconstituted with bone marrow; such mice will develop all hematopoietic cell types except mature T cells.

The recessive *nude* mutation in mice is caused by a mutation in the gene for the transcription factor Wnt, and in homozygous form it causes hairlessness and absence of the thymus. Consequently, these animals fail to develop T cells from bone marrow progenitors. Grafting thymectomized or *nude*/*nude* mice with thymic epithelial elements depleted of lymphocytes allows the graft recipients to develop normal mature T cells. This procedure allows the role of the nonlymphoid thymic stroma to be examined; it has been crucial in determining the role of thymic stromal cells in T-cell development (see Chapter 8).

A-44 *In vivo* depletion of B cells.

There is no single site of B-cell development in mice, so techniques such as thymectomy cannot be applied to the study of B-cell function and development in rodents. However, bursectomy, the surgical removal of the bursa of

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Fabricius in birds, can inhibit the development of B cells in these species. In fact, it was the effect of thymectomy versus bursectomy that led to the naming of T cells for thymus-derived lymphocytes and B cells for bursal-derived lymphocytes. There are no known spontaneous mutations (analogous to the *nude* mutation) in mice that produce animals with T cells but no B cells. However, such mutations exist in humans, leading to a failure to mount humoral immune responses or make antibody. The diseases produced by such mutations are called agammaglobulinemias because they were originally detected as the absence of gamma globulins. The genetic basis for several forms of this disease in humans have now been established (see Section 13-14), and some features of the disease can be reproduced in mice by targeted disruption of the corresponding gene (see Section A-45). Several different mutations in crucial regions of immunoglobulin genes have already been produced by gene targeting and have provided mice lacking B cells.

A-45 Transgenic mice.

The function of genes has traditionally been studied by observing the effects of spontaneous mutations in whole organisms and, more recently, by analyzing the effects of targeted mutations in cultured cells. The advent of gene cloning and *in vitro* mutagenesis now makes it possible to produce specific mutations in whole animals. Mice with extra copies or altered copies of a gene in their genome can be generated by **transgenesis**, which is now a well established procedure. To produce **transgenic mice**, a cloned gene is introduced into the mouse genome by microinjection into the male pronucleus of a fertilized egg, which is then implanted into the uterus of a pseudopregnant female mouse. In some of the eggs, the injected DNA becomes integrated randomly into the genome, giving rise to a mouse that has an extra genetic element of known structure, the transgene (Fig. A.43).

The transgene, to be studied in detail, needs to be introduced onto a stable, well-characterized genetic background. However, it is difficult to prepare transgenic embryos successfully in inbred strains of mice, and transgenic mice are routinely prepared in F_2 embryos (that is, the embryo formed after the mating of two F_1 animals). The transgene must then be bred onto a well-characterized genetic background; this requires 10 generations of backcrossing with an inbred strain to ensure that the integrated transgene is largely (more than 99%) free of heterogeneous genes from the founder mouse of the transgenic mouse line (Fig. A.44).

This technique allows one to study the impact of a newly discovered gene on development, to identify the regulatory regions of a gene required for its normal tissue-specific expression, to determine the effects of its overexpression or expression in inappropriate tissues, and to find out the impact of mutations on gene function. Transgenic mice have been particularly useful in studying the role of T-cell and B-cell receptors in lymphocyte development, as described in Chapter 8.

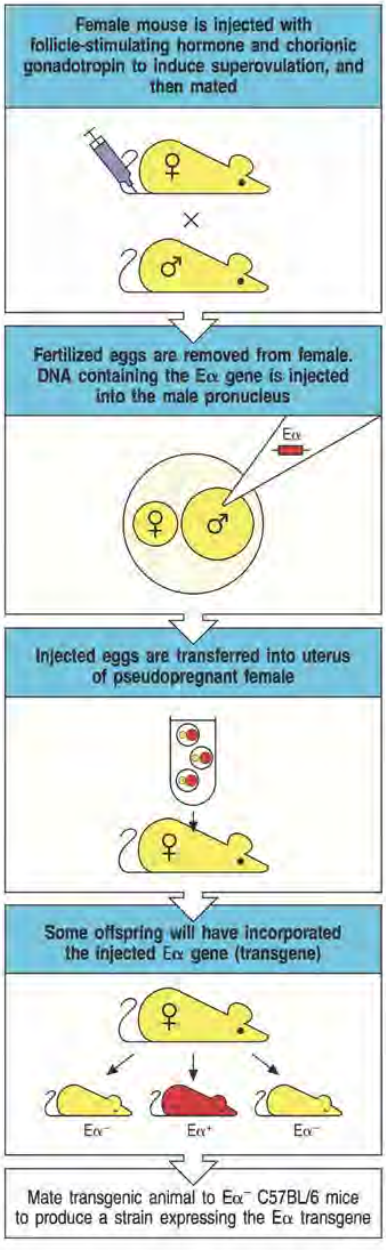


Fig. A.43 The function and expression of genes can be studied *in vivo* by using transgenic mice. DNA encoding a protein of interest, here the mouse MHC class II protein Eα, is purified and microinjected into the male pronuclei of fertilized eggs. The eggs are then implanted into pseudopregnant female mice. The resulting offspring are screened for the presence of the transgene in their cells, and positive mice are used as founders that transmit the transgene to their offspring, establishing a line of transgenic mice that carry one or more extra genes. The function of the Eα gene used here is tested by breeding the transgene into C57BL/6 mice that carry an inactivating mutation in their endogenous Eα gene.

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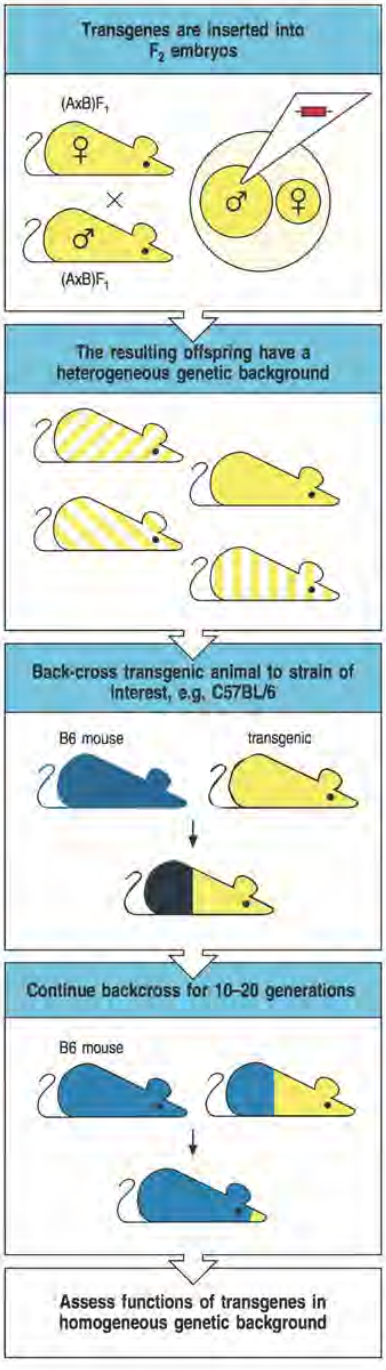


Fig. A.44 The breeding of transgenic co-isogeneic or congenic mouse strains. Transgenic mouse strains are routinely made in F_2 mice. To produce mice on an inbred background, the transgene is introgressively back-crossed onto a standard strain, usually C57BL/6 (B6). The presence of the transgene is tracked by performing PCR on genomic DNA extracted from the tail of young mice. After 10 generations of back-crossing, mice are more than 99% genetically identical, so that any differences observed between the mice are likely to be due to the transgene itself. The same technique can be used to breed a gene knockout into a standard strain of mice, as most gene knockouts are made in the 129 strain of mice (see Fig. A.46). The mice are then intercrossed and homozygous knockout mice are detected by an absence of an intact copy of the gene of interest (as determined by PCR).

A-46 Gene knockout by targeted disruption.

In many cases, the functions of a particular gene can only be fully understood if a mutant animal that does not express the gene can be obtained. Whereas genes used to be discovered through the identification of mutant phenotypes, it is now far more common to discover and isolate the normal gene and then determine its function by replacing it *in vivo* with a defective copy. This procedure is known as **gene knockout**, and it has been made possible by two fairly recent developments: a powerful strategy to select for targeted mutation by homologous recombination, and the development of continuously growing lines of **embryonic stem cells** (**ES cells**). These are embryonic cells that, on implantation into a blastocyst, can give rise to all cell lineages in a chimeric mouse.

The technique of **gene targeting** takes advantage of the phenomenon known as **homologous recombination** (Fig. A.45). Cloned copies of the target gene are altered to make them nonfunctional and are then introduced into the ES cell, where they recombine with the homologous gene in the cell's genome, replacing the normal gene with a nonfunctional copy. Homologous recombination is a rare event in mammalian cells, and thus a powerful selection strategy is required to detect those cells in which it has occurred. Most commonly, the introduced gene construct has its sequence disrupted by an inserted antibiotic-resistance gene such as that for neomycin resistance (*neo*^{r}). If this construct undergoes homologous recombination with the endogenous copy of the gene, the endogenous gene is disrupted but the antibiotic-resistance gene remains functional, allowing cells that have incorporated the gene to be selected in culture for resistance to the neomycin-like drug G418. However, antibiotic resistance on its own shows only that the cells have taken up and integrated the neomycin-resistance gene. To be able to select for those cells in which homologous recombination has occurred, the ends of the construct usually carry the thymidine kinase gene from the herpes simplex virus (*HSV-tk*). Cells that incorporate DNA randomly usually retain the entire DNA construct including *HSV-tk*, whereas homologous recombination between the construct and cellular DNA, the desired result, involves the exchange of homologous DNA sequences so that the nonhomologous *HSV-tk* genes at the ends of the construct are eliminated. Cells carrying *HSV-tk* are killed by the antiviral drug ganciclovir, and so cells with homologous recombinations have the unique feature of being resistant to both neomycin and ganciclovir, allowing them to be selected efficiently when these drugs are added to the cultures (see Fig. A.45).

This technique can be used to produce homozygous mutant cells in which the effects of knocking-out a specific gene can be analyzed. Diploid cells in which both copies of a gene have been mutated by homologous recombination can be selected after transfection with a mixture of constructs in which the gene to be targeted has been disrupted by one or other of two different antibiotic-resistance genes. Having obtained a mutant cell with a functional defect, the defect can be ascribed definitively to the mutated gene if the mutant phenotype can be reverted with a copy of the normal gene transfected into the

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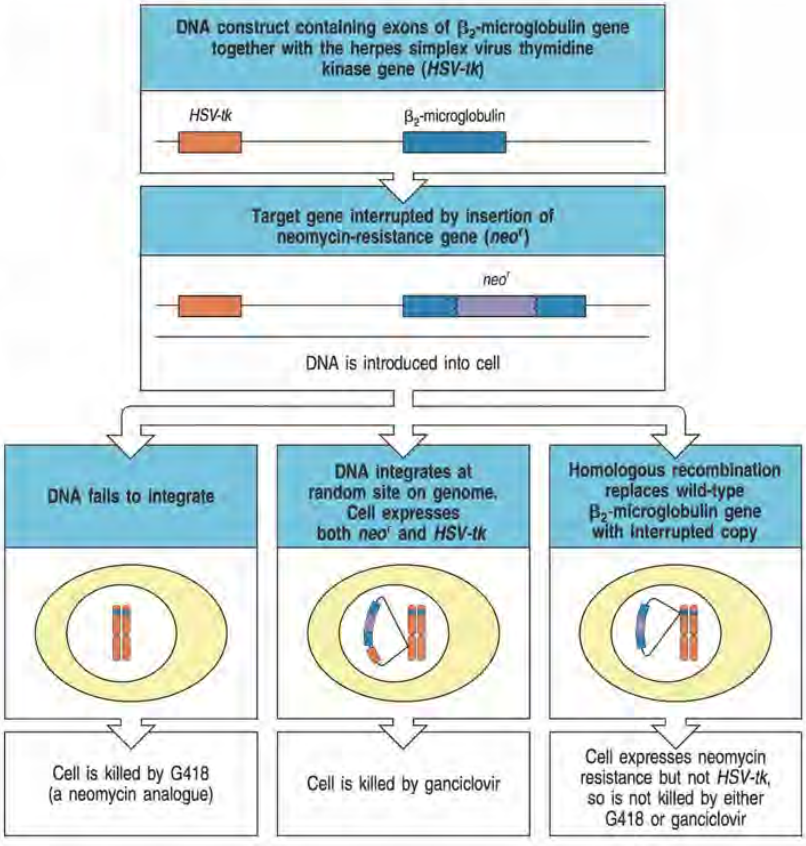


Fig. A.45 The deletion of specific genes can be accomplished by homologous recombination. When pieces of DNA are introduced into cells, they can integrate into cellular DNA in two different ways. If they randomly insert into sites of DNA breaks, the whole piece is usually integrated, often in several copies. However, extrachromosomal DNA can also undergo homologous recombination with the cellular copy of the gene, in which case only the central, homologous region is incorporated into cellular DNA. Inserting a selectable marker gene such as resistance to neomycin (*neo*^{r}) into the coding region of a gene does not prevent homologous recombination, and it achieves two goals. First, any cell that has integrated the injected DNA is protected from the neomycin-like antibiotic G418. Second, when the gene recombines with homologous cellular DNA, the *neo*^{r} gene disrupts the coding sequence of the modified cellular gene. Homologous recombinants can be discriminated from random insertions if the gene encoding herpes simplex virus thymidine kinase (*HSV-tk*) is placed at one or both ends of the DNA construct, which is often known as a 'targeting construct' because it targets the cellular gene. In random DNA integrations, *HSV-tk* is retained. HSV-tk renders the cell sensitive to the antiviral agent ganciclovir. However, as *HSV-tk* is not homologous to the target DNA, it is lost from homologous recombinants. Thus, cells that have undergone homologous recombination are uniquely resistant to both G418 and ganciclovir, and survive in a mixture of the two antibiotics. The presence of the disrupted gene has to be confirmed by Southern blotting or by PCR using primers in the *neo*^{r} gene and in cellular DNA lying outside the region used in the targeting construct. By using two different resistance genes one can disrupt the two cellular copies of a gene, making a deletion mutant (not shown).

mutant cell. Restoration of function means that the defect in the mutant gene has been complemented by the normal gene's function. This technique is very powerful because it allows the gene that is being transferred to be mutated in precise ways to determine which parts of the protein are required for function.

To knock out a gene *in vivo*, it is only necessary to disrupt one copy of the cellular gene in an ES cell. ES cells carrying the mutant gene are produced by targeted mutation (see Fig. A.45) and injected into a blastocyst, which is reimplanted into the uterus. The cells carrying the disrupted gene become incorporated into the developing embryo and contribute to all tissues of the resulting chimeric offspring, including those of the germline. The mutated gene can therefore be transmitted to some of the offspring of the original chimera, and further breeding of the mutant gene to homozygosity produces mice that completely lack the expression of that particular gene product (Fig. A.46). The effects of the absence of the gene's function can then be studied. In addition, the parts of the gene that are essential for its function can be identified by determining whether function can be restored by introducing different mutated copies of the gene back into the genome by transgenesis. The manipulation of the mouse genome by gene knockout and transgenesis is revolutionizing our understanding of the role of individual genes in lymphocyte development and function.

Because the most commonly used ES cells are derived from a poorly characterized strain of mice known as strain 129, the analysis of the function of a gene knockout often requires extensive back-crossing to another strain, just as in transgenic mice (see Fig. A.44). One can track the presence of the mutant copy of the gene by the presence of the *neo*^{r} gene. After sufficient back-crossing, the mice are intercrossed to produce mutants on a stable genetic background.

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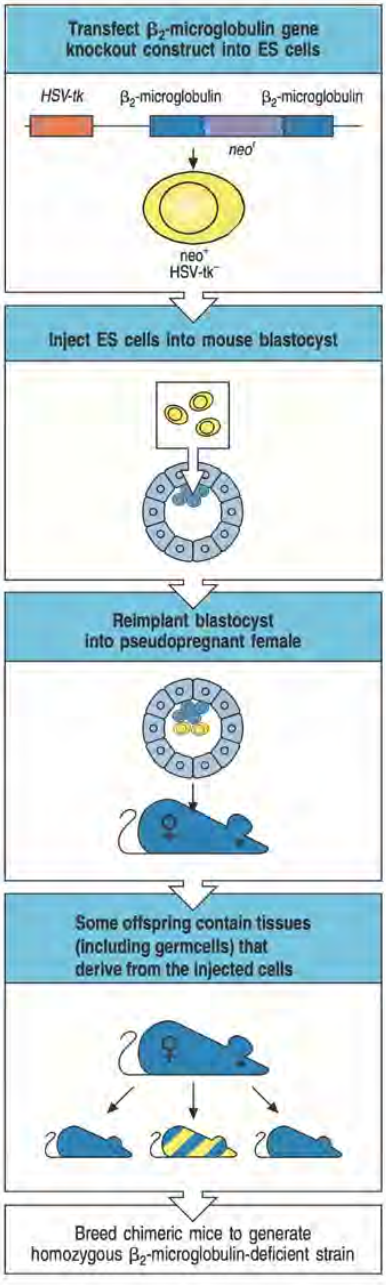


Fig. A.46 Gene knockout in embryonic stem cells enables mutant mice to be produced. Specific genes can be inactivated by homologous recombination in cultures of embryonic stem cells (ES cells). Homologous recombination is performed as described in Fig. A.45. In this example, the gene encoding β_2-microglobulin in ES cells is disrupted by homologous recombination with a targeting construct. Only a single copy of the gene needs to be disrupted. ES cells in which homologous recombination has taken place are injected into mouse blastocysts. If the mutant ES cells give rise to germ cells in the resulting chimeric mice (striped in the figure), the mutant gene can be transferred to their offspring. By breeding the mutant gene to homozygosity, a mutant phenotype is generated. These mutant mice are usually of the 129 strain because gene knockout is generally conducted in ES cells derived from the 129 strain of mice. In this case, the homozygous mutant mice lack MHC class I molecules on their cells, because MHC class I molecules have to pair with β_2-microglobulin for surface expression. The β_2-microglobulin-deficient mice can then be bred with mice transgenic for subtler mutants of the deleted gene, allowing the effect of such mutants to be tested *in vivo*.

A problem with gene knockouts arises when the function of the gene is essential for the survival of the animal; in such cases the gene is termed a **recessive lethal gene**, and homozygous animals cannot be produced. However, by making chimeras with mice that are deficient in B and T cells, it is possible to analyze the function of recessive lethal genes in lymphoid cells. To do this, ES cells with homozygous lethal loss-of-function mutations are injected into blastocysts of mice lacking the ability to rearrange their antigen-receptor genes because of a mutation in their recombinase-activating genes (*RAG* knockout mice). As these chimeric embryos develop, the *RAG*-deficient cells can compensate for any developmental failure resulting from the gene knockout in the ES cells in all except the lymphoid lineage. As long as the mutated ES cells can develop into hematopoietic progenitors in the bone marrow, the embryos will survive and all of the lymphocytes in the resulting chimeric mouse will be derived from the mutant ES cells (Fig. A.47).

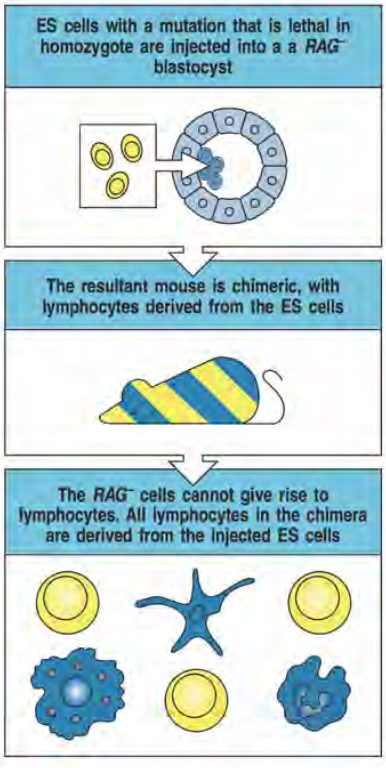


Fig. A.47 The role of recessive lethal genes in lymphocyte function can be studied using *RAG*-deficient chimeric mice. ES cells homozygous for the lethal mutation are injected into a *RAG*-deficient blastocyst (top panel). The *RAG*-deficient cells can give rise to all the tissues of a normal mouse except lymphocytes, and so can compensate for any deficiency in the developmental potential of the mutant ES cells (middle panel). If the mutant ES cells are capable of differentiating into hematopoietic stem cells—that is, if the gene function that has been deleted is not essential for this developmental pathway—then all the lymphocytes in the chimeric mouse will be derived from the ES cells (bottom panel), because *RAG*-deficient mice cannot make lymphocytes of their own.

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A second powerful technique achieves tissue-specific or developmentally regulated gene deletion by employing the DNA sequences and enzymes used by bacteriophage P1 to excise itself from a host cell's genome. Integrated bacteriophage P1 DNA is flanked by recombination signal sequences called *loxP* sites. A recombinase, Cre, recognizes these sites, cuts the DNA and joins the two ends, thus excising the intervening DNA in the form of a circle. This mechanism can be adapted to allow the deletion of specific genes in a transgenic animal only in certain tissues or at certain times in development. First, *loxP* sites flanking a gene, or perhaps just a single exon, are introduced by homologous recombination (Fig. A.48). Usually, the introduction of these sequences into flanking or intronic DNA does not disrupt the normal function of the gene. Mice containing such *loxP* mutant genes are then mated with mice made transgenic for the Cre recombinase, under the control of a tissue-specific or inducible promoter. When the Cre recombinase is active, either in the appropriate tissue or when induced, it excises the DNA between the inserted *loxP* sites, thus inactivating the gene or exon. Thus, for example, using a T-cell specific promoter to drive expression of the Cre recombinase, a gene can be deleted only in T cells, while remaining functional in all other cells of the animal. This extremely powerful genetic technique was used to demonstrate the importance of B-cell receptors in B-cell survival.

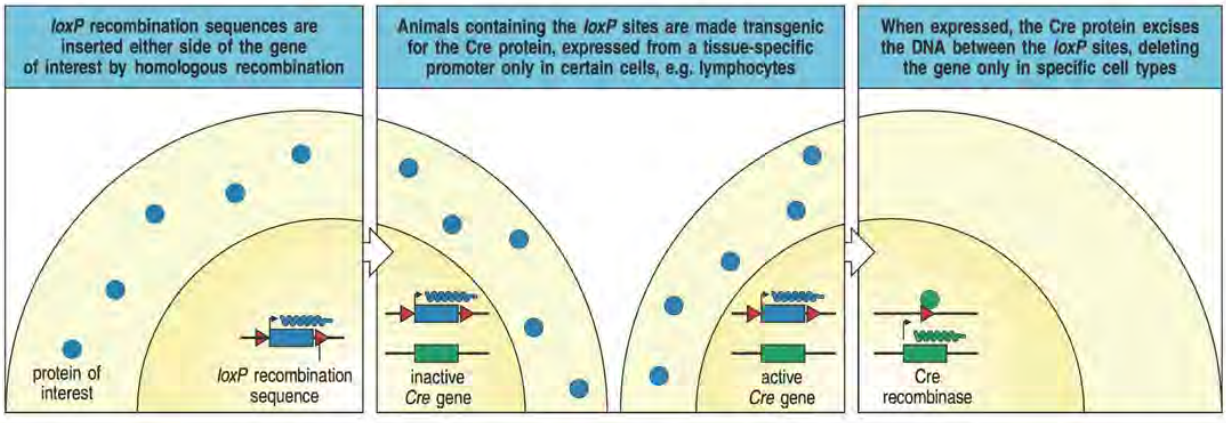


Fig. A.48 The P1 bacteriophage recombination system can be used to eliminate genes in particular cell lineages. The P1 bacteriophage protein Cre excises DNA that is bounded by recombination signal sequences called *loxP* sequences. These sequences can be introduced at either end of a gene by homologous recombination (left panel). Animals carrying genes flanked by *loxP* can also be made transgenic for the gene encoding the Cre protein, which is placed under the control of a tissue-specific promoter so that it is expressed only in certain cells or only at certain times during development (center panel). In the cells in which the Cre protein is expressed, it recognizes the *loxP* sequences and excises the DNA lying between them (right panel). Thus, individual genes can be deleted only in certain cell types or only at certain times. In this way, genes that are essential for the normal development of a mouse can be analyzed for their function in the developed animal and/or in specific cell types. Genes are shown as boxes, RNA as squiggles, and proteins as colored balls.

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