Complementarity, specificity and the nature of epitopes and paratopes in multivalent interactions

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Structural elements of an antibody (Ab) or antigen (Ag) distant from the actual sites mediating contact between Ab and Ag can exert substantial influence on binding to, and discrimination among, multivalent targets. Consequently, multivalent molecules that express the same number of identical binding sites, but that differ in other structural features, can exhibit differences in their ability to discriminate between multivalent ligands. Here, Neil Greenspan and Laurence Cooper review evidence for these effects, and explore implications of the conclusion that effective specificity in multivalent interactions is not completely determined by the degree of complementarity between epitopes and paratopes.

Specificity is a fundamental but multifaceted immunological concept. For instance, Eugene Day identifies three major immunological meanings for the term[1]. First, specificity can be viewed as a measure of the 'goodness of fit' between the antibody (Ab) combining site (paratope) and the antigenic determinant (epitope). Second, specificity can refer to the extent of discrimination between two, or more, monovalent antigens (Ags) mediated by a given Ab. Third, specificity can be used in the context of discrimination between two, or more, multivalent Ags. A fourth sense of specificity may be worthy of consideration, based on observations that the extent of binding of Ab to Ag is not necessarily directly related to the magnitude of the biological effect. For example, comparable amounts of two different Abs, of the same isotype, bound to a cell surface do not always result in comparable amounts of complement activation[2]. Such effects imply that biological specificity can diverge from immunochemical specificity. It is also important to note that biologically relevant discrimination can result in some cases from differences in kinetic constants, as has been suggested for B-cell activation[3], and not only from differences in equilibrium constants.

This article discusses the limitations of the concept of molecular complementarity, with particular reference to data that have bearing on the structural basis for specificity as conceived in the third of the meanings described above. Although the focus of discussion is on discrimination among multivalent Ags by Abs, the chief conclusions may be of broader relevance.

Complementarity and monovalent interactions

Paul Ehrlich's studies on Ab–Ag interactions, together with Emil Fischer's studies on enzyme–substrate interactions, spurred initial development of the notion that two molecules would be likely to bind to one another if they shared complementary surfaces[4]. Linus Pauling, the primary developer of the modern concept of molecular complementarity (or 'complementariness' as described by Pauling), effectively promulgated the notion that it is the degree of complementarity between sites on ligand and receptor that determines the affinity and specificity of the ligand–receptor interaction[5]. Given that this notion has received virtual universal acceptance, it is of interest to note that the degree of complementarity (as operationally defined) between a receptor and a ligand is not always directly related to the free energy of complex formation (or intrinsic affinity)[6]. Similarly, it is difficult to find data documenting that the differential in the free energy of complex formation, for pairings of a receptor with two different ligands, is directly related to the corresponding difference in complementarity (i.e. specificity according to the second of the meanings above). One reason for the failure to document such relationships is that there is no unique method for determining the magnitude of complementarity for a bimolecular complex, even with the availability of a high-resolution crystallographic structure of the complex[7]. Second, complementarity, as determined by any particular scheme of calculation, fails to take full account of one or more of the following factors contributing to the energetics of intermolecular interactions[8]: (1) the energetic effects of rearrangements of solvent molecules and counterions, which remain difficult to calculate[9,10]; (2) the direct contributions of bound water molecules and ions[11]; (3) the conformational alterations of either receptor or ligand associated with complex formation[12]; and (4) the charge-dependent[13] and charge-independent[14] long-range effects. The following discussion will examine data from our own studies and from those of others, which suggest that different receptors with two or more identical binding sites (identical complementarity) do not necessarily bind equivalently to, or express identical specificity for, multivalent targets.

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ALXN2014
IPR2023-01069

ALXN2014
IPR2019-00739

IgG subclass-related discrimination among multivalent Ags

A set of monoclonal Abs (mAbs), known as HGAC 39, with identical variable (V) domains specific for the *N*-acetylglucosamine (GlcNAc) residues of streptococcal group A carbohydrate, the cell-wall polysaccharide of *Streptococcus pyogenes*, have been examined with respect to binding properties. These mAbs, representing the mouse IgG1, IgG2b and IgG3 subclasses, were compared in binding assays both with group A streptococci and with two bacterial strains derived from group A streptococci, as well as with conjugates of GlcNAc covalently coupled to bovine serum albumin (BSA). The strains of streptococci differed in the density of surface GlcNAc residues, and will be referred to as high-, intermediate- and low-epitope-density bacteria. Similarly, the GlcNAc–BSA conjugates differed in GlcNAc-substitution ratio (denoted as $GlcNAc_x$, where x is the substitution number).

Earlier studies established that the IgG3 mAb can bind to multivalent GlcNAc arrays by an Fc-region-dependent cooperative mechanism under conditions where the IgG1 and IgG2b mAbs do not exhibit detectable cooperativity[15–18]. It was also demonstrated that: (1) the IgG3 mAb binds more effectively to the various strains of streptococci, as well as to commercially synthesized $GlcNAc_{16}$–BSA, than the IgG1 and IgG2b mAbs or the IgG3-derived $F(ab')_2$ fragments; and (2) increased amounts of soluble GlcNAc were required to achieve 50% inhibition of the binding of the IgG3 mAb than were required to mediate 50% inhibition of the binding of the IgG1 and IgG2b mAbs or IgG3-derived $F(ab')_2$ fragments. Thus, it was established that differences in the heavy chain constant (C_H) domains, and in the Fc region in particular, substantially influence the apparent functional affinities (avidities) of these IgG mAbs (Ref. 18). In particular, the superior binding of the IgG3 mAb, relative to the IgG1 and IgG2b mAbs, was presumed to stem (at least partially) from the ability of IgG3 mAbs to engage in non-covalent association (IgG3–IgG3), mediated by sites in the Fc region.

Further studies focused on the relative binding patterns of the mAbs and $F(ab')_2$ fragments to bacterial or glycoconjugate Ags displaying different epitope densities. It was observed that the mAbs exhibited different patterns of binding[19,20]; in other words, they differed in their ability to discriminate between the various multivalent Ag targets. For instance, we carried out flow cytometric experiments with the high-, intermediate- and low-epitope-density streptococci in which the different bacteria were mixed with the appropriate Ab species at varying concentrations. Unbound GlcNAc-specific Ab was washed away and, following fixation, the remaining bound Ab was quantitated with fluorescein isothiocyanate (FITC)-conjugated goat Ab specific for mouse Cκ domains. In this assay, the IgG3 mAb bound best to the intermediate-epitope-density strain, while the IgG1 and IgG2b mAbs, and the IgG3-derived $F(ab')_2$ fragments, bound best to the high-epitope-density strain. These results suggest that: (1) the immunochemical identity of multivalent Ags can depend both on epitope organization, or spacing, and on epitope fine structure; (2) for antigenic targets differing in average epitope spacing, the discriminatory abilities of Abs can be influenced by structural differences in the Fc region or other sites residing in the C_H domains. Thus, these results imply that the paratope, as traditionally defined, does not comprise all of the amino acid residues that determine relative binding to different antigenic targets. In this instance, we attribute at least part of the C_H-domain effect on apparent specificity to an influence on the extent of cooperative (noncovalent) interactions between IgG molecules. However, a contribution from structural features that influence segmental flexibility cannot be discounted.

Although, at present, we cannot implicate differences in segmental flexibility as contributing to the subclass-related differences in binding among the GlcNAc-specific mouse mAbs, it has been speculated that such flexibility is crucial for maximizing monogamous, bivalent binding to multivalent targets[21,22]. An implicit consequence of this notion is that differences in segmental flexibility could contribute to differences in relative binding (i.e. specificity according to the third of the meanings described above) for multivalent Ags that vary in epitope density. We are not aware of any systematic study that has addressed this speculation with regard to mouse IgG Abs. However, two recent reports[23,24] have demonstrated differences in binding to multivalent Ags for Abs expressing identical V domains but different human C_H domains. In both cases, the investigators suggested that differences in hinge region structure and segmental flexibility contribute to the binding differences.

The above results suggest that self–nonself discrimination in the effector phase of humoral immunity can depend on the spatial distribution of epitopes, as well as on the fine structure of the epitopes. Indeed, a recent study suggests that differences in the spacing or distribution of a protein epitope on vesicular stomatitis virus (VSV) glycoprotein, displayed on virions as opposed to infected cells, can influence the extent of triggering of cognate B cells by these two immunogens[25]. This result parallels our observations relating to the efferent phase of the immune response, by suggesting that self–nonself discrimination in the afferent phase of the humoral immune response can depend both on epitope distribution and on epitope fine structure.

Specificity and DNA-binding proteins

Several studies of transcription factors have suggested that biochemical and biological specificity for DNA can be influenced by changes in the spacing between the nucleotide sequences physically contacted by protein, as well as by changes in the sequences of the actual contact sites[26,27]. A study of particular importance to this concept is that of Cuenoud and Schepartz, who examined several constructs containing so-called bZIP DNA-recognition domains[28]. These domains contain basic amino acids and are found in proteins containing leucine-zipper dimerization motifs. In this study, the authors examined constructs containing two identical bZIP domains linked through either a leucine-zipper peptide, a disulfide bond or a chelation complex of iron [bis(terpyridyl)iron(II)]. It was found that the molecule

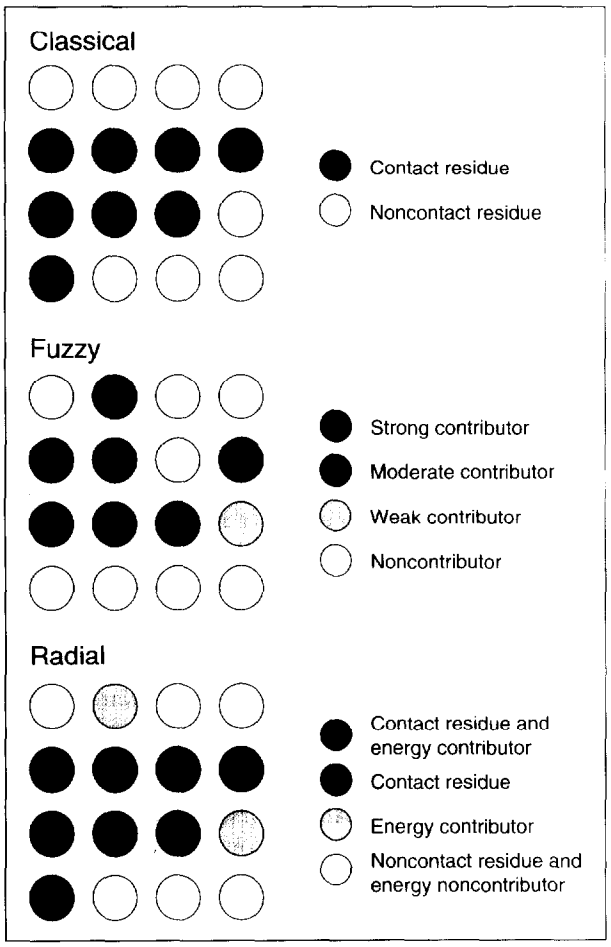

Fig. 1. *Schematic illustration of three different conceptualizations of a paratope as a set of amino acid residues or, alternatively, as a set of atoms. Each circle represents an amino acid residue (atom) in a simplified, hypothetical antibody (Ab). The top and bottom rows correspond to variable module framework residues or amino acid residues in the constant domains, and the middle two rows correspond to hypervariable residues. In the case of the 'classical paratope set', amino acid residues are either part of the paratope or not, depending only on whether the residue in question participates in contacting a given antigen (Ag). Amino acid residues belong to the 'fuzzy paratope set' to different degrees, as indicated by the intensity of the color of each circle. In this case, the degree of membership in the paratope set is correlated with the extent to which each residue contributes to the free energy of complex formation. A similar approach could be based on other variables characterizing the complex, such as the extent of contribution to buried surface area. If the paratope is conceived of as a 'radial' category, then elements of the paratope set include, in this simplified scheme, amino acid residues that contribute to the free energy of complex formation (i.e. stabilize the complex) or that mediate contact between Ab and Ag. Some residues contribute in both senses, others contribute in only one sense or the other. The most realistic concept of a paratope set might include variable degrees of set membership, as well as permit set membership on the basis of only a subset of relevant properties.*

containing the iron complex exhibited strikingly different ratios of binding to cognate and noncognate DNA sites, in comparison with the molecules containing the leucine-zipper motif or the disulfide bond. The cognate sequence differed from the noncognate sequence by the presence of an additional base pair between the two bZIP half-sites.

Thus, in the investigation by Cuenoud and Schepartz, specificity (i.e. discrimination between two different DNA sites) was found to depend on structural features distant from the amino acids that physically contact the DNA, which is analogous to the results with GlcNAc-specific mAbs described above. As the authors state, 'The bis(terpyridyl)iron(II) dimerization domain modifies the inherent selectivity of the GCN4 basic domain without altering its sequence.' Conformational differences in the basic DNA-contacting domains among the three constructs were not formally ruled out in this study. However, these domains are relatively disordered prior to binding to DNA, and the bis(terpyridyl)iron(II)-containing molecule exhibited an increase in α-helical structure typical of bZIP recognition domains upon interaction with cognate DNA. Furthermore, the leucine-zipper-containing and iron-containing molecules gave identical DNase I footprints on cognate DNA, indicating that the DNA-contacting domains for both molecules probably interacted with the DNA in the same manner.

The chief conclusion from the studies described above is that immunological specificity, and molecular specificity in general, can depend on structural features distant from the binding sites (e.g. Ab paratopes) that directly participate in the formation of a bimolecular interface. Since many investigators implicitly regard paratopes and epitopes as the structural correlates of affinity and specificity, it is important to be aware of the potential nonidentity of a site that is defined in terms of intermolecular contact and one that is defined in terms of the structural determinants of affinity or specificity.

While it might seem natural to place primary emphasis on contact residues, each unit of stabilization energy (for complex formation) is equivalent from a thermodynamic perspective, regardless of whether it depends on the identities of amino acids in the contact-mediating regions or in the non-contact-mediating regions of the molecule. As Takeda *et al.*[29] state in a study of the thermodynamics of the interaction between Cro protein and DNA, 'To understand how protein recognizes specific DNA sequences, ..., we must understand the energetics of DNA binding along with the structure because the specificity of DNA association is ultimately determined by the free energy.'

In the context of this last point, an informative recent study examined the thermodynamics of the interaction between *Xenopus laevis* transcription factor IIIA (TFIIIA) and the cognate DNA site from the 5S rRNA gene[30]. TFIIIA contains nine zinc-finger motifs. The authors investigated the thermodynamic consequences of consecutively inactivating, through single amino acid substitutions, each of these nine domains. Measurement of sensitivity to proteolysis of the nine mutant proteins, in comparison with wild-type protein, indicated that the individual fingers were independent structural units. Footprinting by DNase I sensitivity also suggested that the individual zinc-finger domains were largely independent in mediating sequence-specific recognition of DNA. Yet, the binding measurements revealed lower overall affinity of binding for the

intact protein than predicted from the results with the mutants, implying that there were strong, negative (positive change in free energy) thermodynamic interactions among the zinc-finger domains. Current models of the TFIIIA–DNA interaction, based on structural determinations of other zinc-finger proteins, do not provide a ready mechanistic explanation for these results.

Summarizing the above findings with TFIIIA, substitutions for amino acid residues that participate in one interface in a multivalent interaction may exert substantial thermodynamic effects on other contact sites, even when the tertiary structures of the relevant domains are largely independent. A plausible mechanistic explanation for such negative interactions, as exemplified by TFIIIA, is that simultaneous binding to DNA by all nine zinc-finger sites requires the TFIIIA protein to adopt an energetically unfavorable conformation. Thus, it is not necessary to postulate the transmission of a change in tertiary structure from one zinc finger to another in order to account for thermodynamic coupling between recognition sites[31].

Topes and sets

These last points may be restated in terms of set theory. The standard view of paratopes can be formulated in such terms by asserting that a paratope is a set of amino acids or, at a finer level of resolution, a set of atoms. In classical set theory, all the members of a set share a well-defined list of properties, and membership in a set is either 100% or 0%. Most immunologists would probably draw the borders for inclusion of an amino acid residue in a 'paratope set' at the boundaries between the hypervariable regions and the framework regions, or at the boundary between the V and C regions (Fig. 1).

While acknowledging the value of this view, we suggest that paratopes (or epitopes), particularly in the case of multivalent interactions, can be conceived of as sets with imprecise boundaries or membership requirements. This notion of paratopes may bear some analogy to two types of nonclassical sets: radial categories[32] and 'fuzzy sets'[33] (Fig. 1). A radial category is a class of entities with a central or prototype member(s) that possesses all of the attributes that define the category. Other members of the category can share different subsets of such attributes with the prototype(s). A 'fuzzy' set is 'fuzzy' in the sense that the boundaries of set membership are not absolute; membership in a fuzzy set can range from 0%–100% (Ref. 33). Thus, paratopes might be viewed as sets comprising prototype and nonprototype amino acids, or as sets of amino acids with membership values at 100% or <100%. Typical contact-mediating residues in the hypervariable regions, which contribute to the energetics of binding to cognate ligand and to the energetics of discrimination between cognate and noncognate ligands, would represent prototype members of the set. Amino acids in the C domains that influence segmental flexibility[21] or inter-Ab association[34,35], and residues in the V domains that are not contact residues but that determine the canonical conformations of hypervariable loops[36] or otherwise influence contact residues, are possible examples of nonprototype members of the paratope set.

Similar logic can be applied to epitopes. Traditionally, a multivalent Ag such as the cell-wall polysaccharide of group A streptococci is viewed, with respect to mAbs specific for the GlcNAc residues of the cell wall polysaccharide, as GlcNAc epitopes with intervening, inert scaffolding. An alternative perspective is that these intervening structures contribute to the specificity of these epitopes, as 'perceived' by an intact IgG mAb. If these structural features, normally excluded from the epitope set, do not contribute as fully to the affinity or specificity of the interaction with an Ab, then the corresponding structural elements could be viewed as nonprototype members of the set or as having <100% membership in the set.

Typically, immunologists have preconceptions regarding the compartmentalization of a molecule into subunits for the purpose of establishing relationships between structural elements and functions. However, there is no scientific or logical basis for presuming that the structural basis for the 'physiology' of a system can be uniquely defined, especially when the structural elements of the system are highly interactive[37]. The suggestion offered above, to consider paratopes and epitopes in broader terms than is traditionally the case, can be viewed as an alternative decomposition of Ab (or Ag) structure into functional subunits. The key point is that the traditional approach to relating Ab (or Ag) structure and specificity is clearly imperfect for the many biologically relevant multivalent interactions.

Concluding remarks

There are practical implications associated with the notion of paratopes as nonclassical sets of amino acids. For example, we would suggest that, in engineering Ab combining sites, optimization of affinity for monovalent and multivalent interactions may not always result from the same amino acid substitutions. Similar speculations would apply to epitopes and Ags. Thus, such effects could be of relevance for the design either of vaccines or of immunotherapeutic soluble receptors for multivalent targets[34].

The arguments presented above should be considered in a broader context. Virtually any important biological concept, when examined in detail, comprises a family of related concepts that overlap to different degrees and in different ways. Evolutionary biology provides numerous examples of central concepts with several interpretations[38]. Multiple meanings co-exist for concepts such as epitope, paratope or specificity, and which meaning is the most appropriate will be dictated, at least in part, by the nature of the investigational objectives.

We thank David Kaplan and Jon Yewdell for discussion, George Lakoff and David Setzer for critical review of the manuscript, and Aaron Greenspan for assistance in preparing Fig. 1. Supported in part by NIH grant AI-26561.

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