# ANTIGEN PRESENTATION BY CHEMICALLY MODIFIED SPLENOCYTES INDUCES ANTIGEN-SPECIFIC T CELL UNRESPONSIVENESS IN VITRO AND IN VIVO

BY MARC K. JENKINS AND RONALD H. SCHWARTZ

*From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892*

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

Despite a large body of evidence concerning the phenomenon of immune tolerance at the T cell level, the actual mechanism of unresponsiveness is not understood. Experimentally, T cell unresponsiveness can be induced in adults by the intravenous injection of high doses of soluble antigen (1) or of antigen coupled to syngeneic splenocytes (reviewed in references 2–6). Two major hypotheses have been proposed (3–6) to explain unresponsiveness: regulation by suppressor T cells or direct inactivation of responding T cells by antigen.

The suppression model of unresponsiveness states that a complex circuit of interacting suppressor T cells prevent the expression of inducer T cell function. The antigen and MHC specificity of suppression has been shown in most systems to be different from that of inducer T cells, e. g., inducer T cells recognize antigen in association with class II MHC (Ia) molecules, while suppressor T cells often recognize antigen in an unrestricted fashion, or in association with I-J molecules (2, 7, 8). However, several instances of Ia-restricted antigen recognition by suppressor T cells have been described (9, 10). As an alternative to suppression, models of direct T cell inactivation (clonal deletion) state that under certain conditions inducer T cells of the appropriate specificity are functionally or physically deleted after interaction with antigen and Ia molecules (3–6).

Attempts to distinguish between suppression and clonal deletion models have been greatly hampered by the lack of in vitro model systems with which to study the inductive events leading to unresponsiveness. Lamb and coworkers (11) have reported that class II–restricted human T cell clones could be rendered unresponsive in vitro after incubation with free antigen. Although this result suggested that tolerance induction and T cell activation had different specificities, subsequent studies by these investigators (12) showed that unresponsiveness induced in vitro could be blocked by anti–class II antibodies. The relationship of these in vitro observations to the in vivo phenomenon of T cell tolerance induction remained unclear.

To address these issues, we examined the specificity of tolerance induction both in vivo and in vitro using the well-defined response of B10.A mice to pigeon cytochrome *c* as a model system. Previous studies (13) have shown that splenocytes coupled with peptide antigens via the chemical crosslinker 1-ethyl-3-(3-dimethyl-

M. K. Jenkins is a recipient of an Arthritis Foundation postdoctoral fellowship.





JHU 2035
Merck Sharp v. Johns
Hopkins IPR2024-00650

aminopropyl) carbodiimide (ECDI)[1] are tolerogenic in vivo. Our results show that when ECDI-treated splenocytes were used in vitro as APCs, they failed to stimulate proliferation by pigeon cytochrome *c*–specific normal T cell clones and instead induced a state of long-term unresponsiveness that could not be attributed to suppressor T cells. The induction of T cell unresponsiveness in vitro had the same antigen and Ia molecule specificity as T cell activation. Furthermore, as predicted by the in vitro results, T cell unresponsiveness was induced in vivo by the intravenous injection of antigen-coupled splenocytes prepared by ECDI treatment. The induction of this in vivo unresponsiveness also had the same antigen and Ia molecule specificity as T cell activation. The experiments suggested that ECDI treatment alone inactivated an APC function, converting the splenocytes to cells that displayed antigen and Ia molecules, but that induced T cell tolerance instead of proliferation. These results are most consistent with a functional clonal deletion model of T cell tolerance induction and suggest that antigen/Ia molecule recognition under nonmitogenic conditions can result in induction of an unresponsive state.

## Materials and Methods

*Mice.* B10.A/SgSn mice were purchased from The Jackson Laboratory, Bar Harbor, ME. B10.A(4R)/SgSn, B10.A(2R)/SgSn, B10.A(3R), B10.A(5R), B10.A(18R), and C57BL/10Sn (B10) mice were bred in our own colony from Caesarian-derived litters of breeding pairs obtained from Dr. Jack Stimpfling, McLaughlin Research Laboratory, Great Falls, MT. Mice were sex matched within experiments and used between 2–12 mo of age.

*Antigens.* Pigeon cytochrome *c* was purchased from Sigma Chemical Co., St. Louis, MO. Duck cytochrome *c*, prepared as described (14), was the kind gift of Dr. E. Margoliash, Northwestern University, Evanston, IL. Pigeon cytochrome *c* fragments 1-65, 66-80, and 81-104 were prepared by cyanogen bromide cleavage and were purified on a Sephadex G50 superfine column (Pharmacia Fine Chemicals, Piscataway, NJ) (15). An analog of moth cytochrome *c* fragment 93-103 containing a glutamine for lysine substitution at position 99 and a glutamic acid for aspartic acid substitution at position 93, moth 93-103(93E,99Q), was synthesized by Dr. Barbara Fox (National Institute of Allergy and Infectious Diseases, Bethesda, MD) using the Merrifield solid-phase procedure as previously described (16). Another analog of moth cytochrome *c* containing a glutamic acid for aspartic acid substitution at position 93 and an alanine for leucine substitution at position 98, moth 86-89;93-103(93E,98A)[2] was prepared by the Merrifield solid-phase procedure and was kindly provided by Dr. B. Singh (Dept. of Immunology, University of Alberta, Canada). PPD was purchased from Connaught Laboratories Ltd., Willowsdale, Ontario, Canada. GAT was purchased from Vega Biotechnologies, Inc., Tucson, AZ.

*Preparation of ECDI-treated Splenocytes.* Erythrocyte-free splenocytes were treated with ECDI as previously described (13). Briefly, $10^8$ spleen cells were incubated for 1 h on ice in 0.44 ml of 0.9% NaCl containing 75 mM ECDI (Calbiochem-Behring Corp., La Jolla, CA). The cells were washed extensively in serum-free RPMI 1640 medium to stop the coupling reaction. In some experiments, splenocytes were fractionated into T cell–enriched or B cell and macrophage-enriched populations before ECDI treatment. T cell–enriched populations were obtained by treating nylon-wool nonadherent (17) B10.A splenocytes with 14.4.4S (anti-$E^k_\beta$:$E^k_\alpha$, reference 18) and 10.2.16 (anti-$A^k_\beta$:$A^k_\alpha$; reference 19) mAbs (1:1000 ascites) for 1 h on ice, followed by a 30-min incubation in a 1:4 dilution of guinea pig complement (Gibco Laboratories, Grand Island, NY) at 37°C. These cells

[1] *Abbreviation used in this paper:* ECDI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

[2] Fox, B. S., C. Chen, E. Fraga, C. French, B. Singh, and R. H. Schwartz. 1986. Evaluation of the trimolecular complex model of T cell recognition. Manuscript in preparation.

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

responded well to Con A in the presence of irradiated splenocytes but failed to respond to LPS (data not shown). B cell plus macrophage-enriched populations were obtained by treating B10.A splenocytes with 31.11 (anti-Thy-1, reference 20), 53-7.3 (anti-Ly-1, reference 21), 53-6.7 (anti-Lyt-2, reference 21), and MAR 18.5 (mouse anti–rat Ig, reference 22) mAbs (1:10 culture supernatants) plus complement. These cells failed to respond to Con A but responded well to LPS (data not shown). For the in vivo experiments, peptide antigens were directly coupled to B10.A, B10.A(3R), B10.A(5R), B10.A(18R), or B10.A(4R) splenocytes as described above except that the reaction mixture contained 400 $\mu$M antigen and 75 mM ECDI. Where indicated, B10.A splenocytes were depleted of cells bearing Ia molecules as described above for preparation of T cell–enriched populations, just before antigen coupling. Trace labeling studies indicated that 0.5 $\mu$g of pigeon cytochrome *c* was coupled to $10^7$ splenocytes (data not shown).

*T Cell Clones.* Normal T cell clones A.E7 (23) and F1.A.2 (24) were derived from pigeon cytochrome *c*–immunized B10.A and (B10.A(3R) × B10.A)$F_1$ mice, respectively. Normal T cell clone 3R.3.11 (25) was derived from B10.A(3R) mice immunized with pigeon cytochrome *c* fragment 1-65. All three normal clones express the L3T4$^+$, Lyt-2$^-$ surface phenotype. T cell clones were routinely maintained by a modification of the Kimoto and Fathman (26) rest-stimulation protocol as described by Ashwell et al. (27). For the in vitro tolerance experiments, T cell clones were maintained by weekly addition of only IL-2-containing supernatants (25–50 U/ml) for 1 mo after the last stimulation with antigen and irradiated splenocytes (3,000 rad). The few surviving splenocytes were then depleted by anti–Ia molecule plus complement treatment: 10.2.16 and 14.4.4S for A.E7 and F1.A.2, or M5/114 (anti-$A^b_\beta$:$A^b_\alpha$, reference 28) for 3R.3.11. Dead cells were removed on Ficoll-Hypaque density gradients (Pharmacia Fine Chemicals). After this treatment, T cell clones failed to respond to high doses of antigen plus allogeneic splenocytes, indicating complete depletion of syngeneic APC.

*T Cell Clone Proliferation Assay.* Cloned T cells ($2 \times 10^4$) were cultured in 0.2 ml of Click's medium supplemented with 10% heat-inactivated FCS, $2 \times 10^{-3}$ M glutamine, $5 \times 10^{-5}$ M 2-ME, and antibiotics in 96-well microtiter plates (Costar No. 3596; Costar, Cambridge, MA) with $5 \times 10^5$ irradiated (3,000 rad) syngeneic splenocytes and varying doses of antigen or with IL-2 alone (100 U/ml). Where indicated, cultures contained $5 \times 10^5$ ECDI-treated splenocytes as APC. After 2 d of culture, 1 $\mu$Ci of [$^3$H]thymidine (6.7 mCi/mM, New England Nuclear, Boston, MA) was added to each well. 16 h later, the cells were harvested using a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA). Thymidine incorporation into DNA was quantitated by liquid scintillation counting. Determinations were performed in duplicate with the results expressed as $\Delta$ cpm, i.e., (mean cpm in antigen stimulated wells) − (mean cpm in wells without antigen). Background proliferation (no antigen) was routinely <1,000 cpm.

*Induction of T Cell Clone Unresponsiveness In Vitro.* T cell clones ($5 \times 10^5$) were preincubated overnight in Costar 24-well plates (No. 3524) in 2.0 ml of medium containing $5 \times 10^6$ ECDI-treated splenocytes with or without soluble antigen. In some experiments, purified 14.4.4S or 10.2.16 mAbs (1 $\mu$g/ml, kindly provided by Dr. A. Kruisbeek, NIH) were added to the preincubation cultures. The cloned T cells were recovered the next day on Ficoll-Hypaque density gradients, washed extensively in RPMI 1640, and restimulated ($2 \times 10^4$) with fresh, irradiated splenocytes ($5 \times 10^5$) and antigen in 96-well microtiter plates, as described above for the T cell clone proliferation assay. Recovery from preincubation cultures was routinely 60–80% of the input cell number. T cell clones preincubated in the absence of antigen with ECDI-treated or normal splenocytes responded similarly to restimulation, suggesting that the ECDI-treated cells were not toxic (data not shown).

*Kinetics and Duration of T Cell Clone Unresponsiveness Induced In Vitro.* For the experiments shown in Fig. 6, A.E7 cells ($5 \times 10^5$) were incubated with $5 \times 10^6$ ECDI-treated B10.A splenocytes in 2.0 ml medium with or without 5 $\mu$M pigeon fragment 81-104 for 2, 5, or 16 h. At the indicated times, A.E7 cells were separated from the treated APC and antigen on density gradients, washed extensively, and restimulated with normal APC and antigen as described above. For the experiments shown in Fig. 7, F1.A.2 cells

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

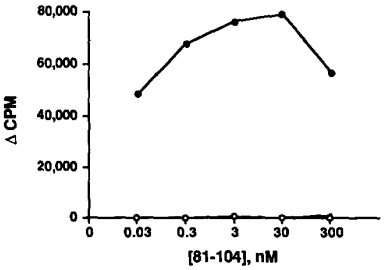


FIGURE 1. ECDI-treated splenocytes fail to stimulate proliferation by a normal T cell clone. $5 \times 10^5$ ECDI-treated (*open circles*) or 3,000 rad irradiated normal (*filled circles*) B10.A splenocytes were cultured with $2 \times 10^4$ F1.A.2 normal cloned T cells and the indicated doses of pigeon fragment 81-104. Proliferation by F1.A.2 was determined by [$^3$H]thymidine incorporation with the results expressed as Δ cpm.

($5 \times 10^5$) were incubated overnight with $5 \times 10^6$ ECDI-treated B10.A splenocytes in 2.0 ml medium with or without 1 μM pigeon fragment 81-104. The T cells were recovered, washed, and $2 \times 10^4$ cell were placed in 0.1 ml medium in 96-well microtiter plates. At later times, $5 \times 10^5$ irradiated B10.A splenocytes and various doses of pigeon fragment 81-104 or IL-2 alone were added in 0.1 ml of medium. Restimulation with normal APC and antigen was determined as described above.

*Induction of T Cell Unresponsiveness In Vivo.* Mice were injected intravenously with 0.5 ml RPMI 1640 containing either $2 \times 10^7$ or $5 \times 10^7$ antigen- or sham-coupled splenocytes. 1 or 4 days after injection, recipient mice were immunized subcutaneously in each footpad and at the base of the tail with a total of 10 nmol (in 0.2 ml) of pigeon cytochrome *c* or moth 86-89;93-103(93E,98A) emulsified in CFA containing *Mycobacterium tuberculosis* H37Ra (Difco Laboratories Inc., Detroit, MI).

*Lymph Node T Cell Proliferation Assay.* 7 d after priming, draining lymph node T cells were prepared by passage over nylon-wool columns (17). T cells ($4 \times 10^5$ cells/well) were cultured in 96-well microtiter plates with varying doses of pigeon cytochrome *c* fragment 81-104, moth 86-89; 93-103(93E,98A), or PPD, and $10^5$ irradiated (3,000 rad) syngeneic spleen cells in 0.2 ml of medium. Cultures were pulsed with [$^3$H]thymidine on day 3, harvested on day 4, and thymidine incorporation was determined as described above. Determinations were performed in triplicate and the results are expressed as Δ cpm ± SEM calculated as the square root of the sum of the squares of the individual errors.

## Results

*Antigen Presentation by ECDI-treated Splenocytes to T Cell Clones In Vitro.* Previous analyses (13) have revealed that specific T cell unresponsiveness can be induced in vivo by the intravenous injection of syngeneic splenocytes coupled with protein antigens by the chemical crosslinker ECDI. To gain a better understanding of this unresponsiveness, we examined the antigen presentation capacity of ECDI-treated splenocytes in vitro. Normal B10.A T cell clones proliferate in response to the COOH-terminal cyanogen bromide fragment of pigeon cytochrome *c* (residues 81-104) in association with the $E^k_\beta$:$E^k_\alpha$ Ia molecule expressed on APC (29). However, a clone with this specificity (F1.A.2) failed to proliferate in response to B10.A splenocytes coupled with pigeon fragment 81-104 by ECDI (data not shown), or to ECDI-treated B10.A splenocytes and soluble pigeon fragment 81-104 (Fig. 1). In contrast, normal B10.A splenocytes and pigeon fragment 81-104 stimulated a vigorous proliferative response.

Several observations suggested that the inability of ECDI-treated APC to

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

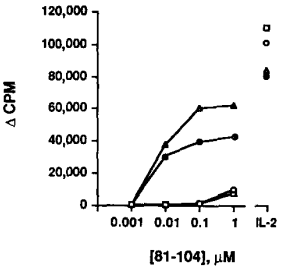


FIGURE 2. Induction of T cell unresponsiveness in vitro. Cloned T cell A.E7 ($5 \times 10^5$) was incubated overnight with various ECDI-treated B10.A spleen cell populations with or without pigeon fragment 81-104. T cell–enriched and B cell plus macrophage–enriched populations were prepared as described in Materials and Methods. The A.E7 cells were reisolated the next day and cultured ($2 \times 10^4$) with the indicated antigen doses and $5 \times 10^5$ irradiated B10.A spleen cells or IL-2-containing supernatants (100 U/ml) alone. Proliferation of restimulated cells was measured by [$^3$H]thymidine incorporation and the results are expressed as Δ cpm. A.E7 preincubation conditions: ECDI-treated B10.A unfractionated splenocytes with (*open circles*) or without (*filled circles*) 5 μM pigeon fragment 81-104, or ECDI-treated B10.A T cells plus 5 μM pigeon fragment 81-104 (*open triangles*) or ECDI-treated B10.A B cells and macrophages plus 5 μM fragment 81-104 (*open squares*).

stimulate proliferation was probably not the result of extensive Ia molecule modification. First, ECDI-treated splenocytes had Ia-restricted effects on normal T cell clones that could be blocked with anti-Ia mAbs (see below). Second, ECDI-treated APC expressed relatively normal levels of Ia molecules, as detected by mAbs (data not shown). Finally, ECDI-treated APC could weakly stimulate IL-2 production by the 2B4 T cell hybridoma (data not shown); this cell requires only pigeon fragment 81-104 and $E^k_\beta$:$E^k_\alpha$ molecules for activation, as demonstrated by studies using purified Ia molecules incorporated into planar membranes (29a). These observations are most consistent with the interpretation that ECDI treatment inactivated an accessory function of the APC that is necessary to stimulate proliferation by a normal T cell clone.

*The Induction of T Cell Unresponsiveness In Vitro.* The observation that ECDI-treated APC expressed functional Ia molecules and yet failed to stimulate normal T cell clones to proliferate raised the possibility that recognition of antigen on ECDI-treated APC resulted in unresponsiveness. To test this, the normal T cell clone A.E7 (specific for pigeon fragment 81-104 and $E^k_\beta$:$E^b_\alpha$) was preincubated overnight with various ECDI-treated B10.A spleen cell populations, with or without antigen, and then was restimulated with antigen and untreated syngeneic APC (Fig. 2). A.E7 cells preincubated with pigeon fragment 81-104 and either ECDI-treated B10.A unfractionated splenocytes or ECDI-treated B10.A B cells plus macrophages were almost completely unresponsive to restimulation with untreated B10.A splenocytes and pigeon fragment 81-104. These cells responded well to exogenous IL-2, indicating that cell death did not account for antigen/Ia molecule unresponsiveness. In contrast, A.E7 cells preincubated with ECDI-treated B10.A splenocytes in the absence of antigen or with ECDI-treated B10.A T cells plus pigeon fragment 81-104 responded well to restimulation with untreated APC and antigen. These results demonstrated that antigen presentation in vitro by ECDI-treated splenocytes results in T cell unresponsiveness. In

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

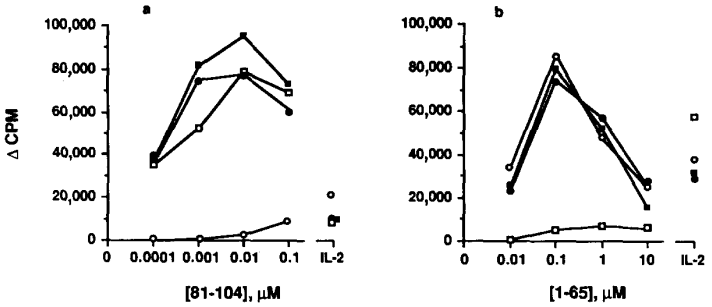


FIGURE 3. MHC specificity of T cell unresponsiveness induced in vitro. Cloned T cells ($5 \times 10^5$) A.E7 (*a*) or 3R.3.11 (*b*) were incubated overnight with $5 \times 10^6$ ECDI-treated splenocytes with or without antigen. The T cells were reisolated and recultured ($2 \times 10^4$) with the indicated antigens, and $5 \times 10^5$ B10.A (for A.E7) or B10 (for 3R.3.11) irradiated spleen cells or IL-2-containing supernatants (100 U/ml) alone. Proliferation of restimulated cells was measured by [$^3$H]thymidine incorporation. A.E7 preincubation conditions: ECDI-treated B10.A splenocytes with (*open circles*) or without (*filled circles*) 0.5 μM pigeon fragment 81-104, or ECDI-treated B10 splenocytes with (*open squares*) or without pigeon fragment 81-104 (*filled squares*). (*b*) 3R.3.11 preincubation conditions: ECDI-treated B10 splenocytes with (*open squares*) or without pigeon fragment 1-65 (*filled squares*), or ECDI-treated B10.A splenocytes with (*open circles*) or without pigeon fragment 1-65 (*filled circles*).

addition, the critical population in spleen responsible for the induction of unresponsiveness was ECDI-treated Ia$^+$ cells (B cells plus macrophages) and not ECDI-treated T cells, strongly arguing against a role for suppressor T cells in this phenomenon.

*Specificity of the Induction of T Cell Unresponsiveness In Vitro.* It has been demonstrated (8, 29) that inducer T cell activation requires recognition of antigen in association with MHC molecules. The experiments shown in Fig. 3 were performed to determine if a similar restriction governed the induction of T cell unresponsiveness in vitro. A.E7 cells (specific for fragment 81-104 and $E^k_\beta$:$E^k_\alpha$) preincubated with ECDI-treated B10.A splenocytes and pigeon fragment 81-104 were almost completely unresponsive to restimulation with normal B10.A splenocytes and pigeon fragment 81-104 (Fig. 3*a*). Unresponsiveness was not induced after preincubation with ECDI-treated B10.A splenocytes alone or with ECDI-treated B10 splenocytes bearing $A^b_\beta$:$A^b_\alpha$ class II molecules with or without pigeon fragment 81-104. In the reciprocal experiment, clone 3R.3.11 (specific for pigeon fragment 1-65 and $A^b_\beta$:$A^b_\alpha$) was rendered unresponsive to restimulation with B10 splenocytes and pigeon fragment 1-65 by preincubation with ECDI-treated B10 splenocytes and fragment 1-65, but not ECDI-treated B10 splenocytes alone or ECDI-treated B10.A splenocytes with or without antigen (Fig. 3*b*). Both A.E7 and 3R.3.11 cells that were rendered unresponsive to antigen and Ia molecule restimulation responded to exogenous IL-2, demonstrating that cell death did not account for unresponsiveness. These results show that the induction of T cell unresponsiveness in vitro is MHC-restricted.

The specificity of unresponsiveness induced in vitro was further examined as shown in Fig. 4. F1.A.2 cells were rendered unresponsive to restimulation with B10.A APC and pigeon fragment 81-104 after preincubation with ECDI-treated B10.A splenocytes and pigeon fragment 81-104, but not with moth 93-

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

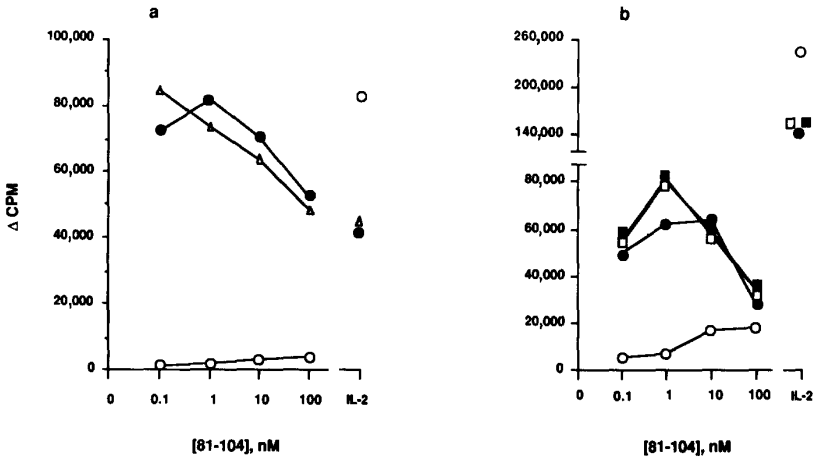


FIGURE 4. Antigen and Ia molecule specificity of T cell unresponsiveness induced in vitro. T cell clone F1.A.2 was preincubated with ECDI-treated splenocytes with or without antigen as described in the legend to Fig. 2. Proliferation of restimulated cells was measured by [$^3$H]-thymidine incorporation. F1.A.2 preincubation conditions: ECDI-treated B10.A splenocytes with (*open circles*) or without (*filled circles*) 1.0 μM pigeon fragment 81-104 (*a* and *b*), or ECDI-treated B10.A splenocytes with 1.0 μM moth peptide 93-103(93E,99Q) (*a*, *open triangles*), or ECDI-treated B10.A(4R) splenocytes with (*b*, *open squares*) or without (*b*, *filled squares*) 1.0 μM pigeon fragment 81-104.

103(93E,99Q), a nonstimulatory peptide analog containing the moth cytochrome *c* sequence with a glutamine for lysine substitution at residue 99 (29) (Fig. 4*a*). Similarly, preincubation of F1.A.2 with pigeon fragment 81-104 and ECDI-treated B10.A(4R) splenocytes, which do not express $E_\beta$:$E_\alpha$ molecules, did not result in unresponsiveness (Fig. 4*b*). Furthermore, preincubation of A.E7 with ECDI-treated B10.A splenocytes and pigeon fragment 81-104, in the presence of anti-$E^k_\beta$:$E^k_\alpha$ but not anti-$A^k_\beta$:$A^k_\alpha$ mAbs, completely blocked the induction of unresponsiveness in vitro (Table I), demonstrating that the Ia molecules present on ECDI-treated splenocytes are required for the induction of the unresponsive state. In all cases, unresponsiveness could not be accounted for by cell death, as the clones proliferated well in response to exogenous IL-2 after preincubation with the appropriate antigen and ECDI-treated APC. These results demonstrate that the induction of T cell unresponsiveness in vitro has the identical antigen and Ia molecule specificity as the induction of the T cell clone's proliferative response.

*Dependence of the Induction of Unresponsiveness on the Antigen Concentration and the Number of ECDI-Treated APC.* The quantitative aspects of the induction of unresponsiveness are shown in Fig. 5. A.E7 preincubated with a fixed number of ECDI-treated B10.A splenocytes and increasing concentrations of pigeon fragment 81-104 (Fig. 5*a*), or with a fixed concentration of pigeon fragment 81-104 and increasing numbers of ECDI-treated B10.A splenocytes (Fig. 5*b*), were progressively less responsive to restimulation. Maximal effects were observed with 1–10 μM of antigen and $5 \times 10^6$ ECDI-treated splenocytes.

*Kinetics of Induction of T Cell Unresponsiveness In Vitro.* A.E7 cells were

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

TABLE I

*Effect of Anti-Ia mAbs on the Induction of T Cell Unresponsiveness In Vitro*

| Group | Preincubation conditions* | | | | Restimulation response (Δ cpm)‡ to: | |
|---|---|---|---|---|---|---|
| | T cell clone | APC | Antigen | Antibody | B10.A APC + 81-104 | IL-2 |
| A | A.E7 | B10.A-ECDI | — | — | 95,000 | 110,000 |
| B | A.E7 | B10.A-ECDI | 81-104 | — | 2,500 (97)§ | 125,000 |
| C | A.E7 | B10.A-ECDI | 81-104 | 14.4.4S (anti-$E^k_\beta$:$E^k_\alpha$) | 87,500 (8) | 105,000 |
| D | A.E7 | B10.A-ECDI | 81-104 | 10.2.16 (anti-$A^k_\beta$:$A^k_\alpha$) | 3,000 (97) | 155,000 |

* A.E7 cells ($5 \times 10^5$) were preincubated overnight with $5 \times 10^6$ ECDI-treated B10.A splenocytes without antigen (group A) or with $5 \times 10^6$ ECDI-treated B10.A splenocytes and 5 μM pigeon fragment 81-104 without (group *B*) or with 1 μg/ml 14.4.4S (group C) or 10.2.16 (group D) mAbs.

‡ After preincubation, A.E7 cells were reisolated and cultured ($2 \times 10^4$) with $5 \times 10^5$ B10.A irradiated splenocytes with or without 0.01 μM pigeon fragment 81-104 or 100 U of IL-2 alone. The results are expressed as Δ cpm.

§ Numbers in parentheses show percent inhibition of the proliferative response compared with group A.

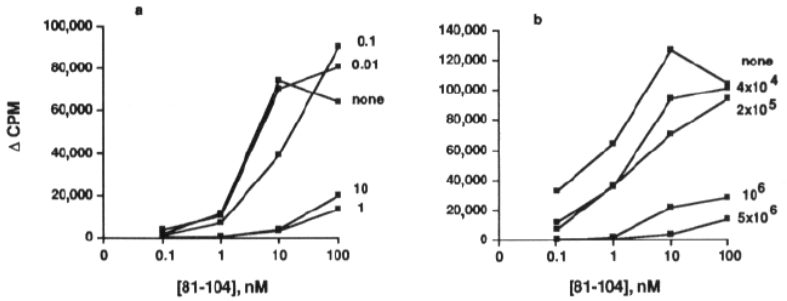


FIGURE 5. Dose dependency of T cell unresponsiveness on antigen concentration and APC number. T cell clone A.E7 ($5 \times 10^5$) was preincubated overnight with $5 \times 10^6$ ECDI-treated B10.A splenocytes and the indicated concentrations (μM) of pigeon fragment 81-104 (*a*) or with 1 μM pigeon fragment 81-104 and the indicated numbers of ECDI-treated B10.A splenocytes (*b*). The T cells were reisolated and restimulated as described in the legend to Fig. 2. The results are expressed as Δ cpm.

preincubated with ECDI-treated B10.A splenocytes with or without pigeon fragment 81-104 for various periods of time to determine the time course for the induction of unresponsiveness in vitro (Fig. 6). Unresponsiveness to restimulation with normal APC and antigen was apparent after only 2 h, was partially induced after 5 h, and was completely induced after 16 h of preincubation. Thus, unresponsiveness was not totally induced immediately, but required >5 h to achieve a maximal effect.

*Duration of T Cell Unresponsiveness Induced In Vitro.* The duration of in vitro–induced unresponsiveness was tested by preincubating F1.A.2 cells overnight with ECDI-treated B10.A splenocytes with or without pigeon fragment 81-104, followed by restimulation 2, 4, 6, or 8 d later (Fig. 7). At all time points, F1.A.2 cells were completely unresponsive to restimulation with untreated B10.A splenocytes and antigen, although they remained responsive to IL-2 (data not shown). Thus, as reported for unresponsiveness induced in vivo by antigen-coupled cells

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

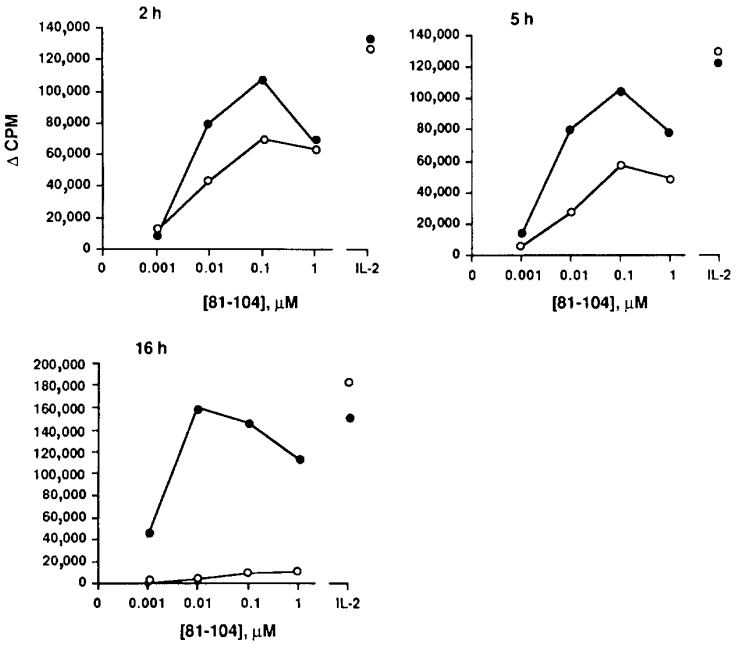


FIGURE 6. Time course of the induction of T cell unresponsiveness in vitro. T cell clone A.E7 ($5 \times 10^5$) was preincubated with $5 \times 10^6$ ECDI-treated B10.A splenocytes with (*open circles*) or without (*filled circles*) 5 μM pigeon fragment 81-104 for the indicated times, after which the T cells were reisolated and restimulated as described in the legend to Fig. 2. The results are expressed as Δ cpm.

(30), unresponsiveness induced in vitro appears to last for a relatively long period of time.

*Specificity of T Cell Unresponsiveness Induced In Vivo.* Our studies demonstrate that ECDI-treated splenocytes induce T cell unresponsiveness in vitro. In addition, others have shown (3–5, 13) that intravenous injection of antigen-coupled splenocytes results in T cell unresponsiveness in vivo. Based on these observations, we examined the fine specificity of T cell unresponsiveness to pigeon cytochrome *c* induced in vivo. B10.A mice were injected intravenously with B10.A splenocytes coupled with antigen via ECDI. 4 d later, recipient mice were immunized in the footpads with pigeon cytochrome *c* emulsified in CFA. As shown in Fig. 8, T cells from recipients of splenocytes coupled via ECDI with intact pigeon cytochrome *c* or pigeon fragment 81-104 proliferated very poorly in response to fragment 81-104 (Fig. 8*a*), although these cells responded normally to PPD present in the adjuvant used for immunization (Fig. 8*c*). Control T cells from mice receiving splenocytes treated with ECDI in the absence of antigen, proliferated strongly in response to pigeon fragment 81-104 (Fig. 8*a*). The intact molecule was a better tolerogen than the 81-104 fragment, possibly because there also exist B10.A T cells that are specific for pigeon fragment 1-65 in association with $A^k_\beta$:$A^k_\alpha$ (25); these may have facilitated the survival and/or expansion of T cells specific for fragment 81-104 and $E^k_\beta$:$E^k_\alpha$.

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

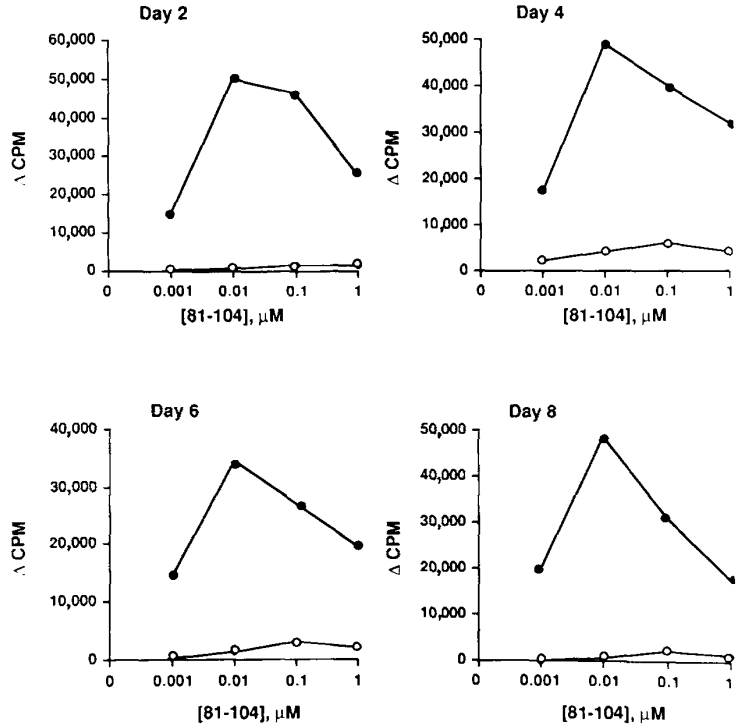


FIGURE 7. Duration of T cell unresponsiveness induced in vitro. T cell clone F1.A.2 ($5 \times 10^5$/well) was preincubated with ECDI-treated B10.A splenocytes ($5 \times 10^6$/well) with (*open circles*) or without (*filled circles*) 1.0 μM pigeon fragment 81-104. The T cells were reisolated, placed in microtiter plates ($2 \times 10^4$), and restimulated by the addition of various antigen concentrations and $5 \times 10^5$ irradiated B10.A splenocytes 2, 4, 6, or 8 d later. The results are expressed as Δ cpm.

The antigen specificity of the tolerance induction was similar to that of proliferation because B10.A splenocytes coupled with pigeon fragment 66-80 or intact duck cytochrome *c* (neither of which stimulate a proliferative response in B10.A mice, reference 31) failed to induce hyporesponsiveness in vivo (Fig. 8*a*). A requirement for Ia molecules was also demonstrated, as B10.A splenocytes that were depleted of cells bearing Ia molecules and then coupled with pigeon cytochrome *c* were poor tolerogens in vivo (Fig. 8*b*). Furthermore, pigeon 81-104-coupled B10.A(4R) splenocytes ($K^k$, $A^k_\beta$, $A^k_\alpha$, $E^k_\beta$, $E^b_\alpha$, $D^b$), which do not express $E_\beta$:$E_\alpha$ molecules, failed to induce fragment 81-104-specific T cell unresponsiveness in pigeon cytochrome *c*–primed B10.A ($K^k$, $A^k_\beta$, $A^k_\alpha$, $E^k_\beta$, $E^k_\alpha$, $D^d$) mice (Fig. 9, *a–c*). This suggests that the $E^k_\beta$:$E^k_\alpha$ Ia molecule is necessary for the tolerance induction. An alternative explanation for this last result, however, is that allogeneic effects in vivo due to the H-2D region disparity between B10.A and B10.A(4R) obscured the tolerance induction. To eliminate this possibility the experiment was repeated using B10.A(2R) ($K^k$, $A^k_\beta$, $A^k_\alpha$, $E^k_\beta$, $E^k_\alpha$, $D^b$) recipients and fragment 81-104, ECDI-coupled B10.A(2R) (tolerogenic) vs. B10.A(4R) (non-

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

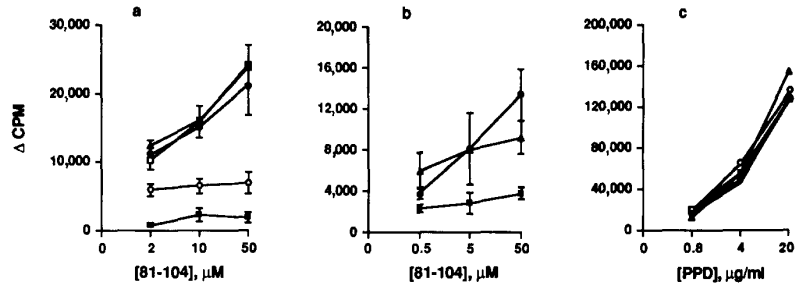


FIGURE 8. Antigen specificity of unresponsiveness induced in vivo by ECDI-treated splenocytes. B10.A mice (two per group) were injected intravenously with $2 \times 10^7$ ECDI-treated B10.A splenocytes coupled with pigeon cytochrome *c* (*closed squares* in *a–c*), pigeon fragment 81-104 (*open circles* in *a* and *c*), pigeon fragment 66-80 (*open squares* in *a* and *c*), duck cytochrome *c* (*open triangles* in *a* and *c*), nothing (*filled circles* in *a–c*), or $2 \times 10^7$ B10.A splenocytes depleted of cells bearing Ia molecules and then coupled with pigeon cytochrome *c* (*filled triangles* in *b*). 4 d later, recipient mice were immunized with pigeon cytochrome *c* in CFA. 7 d after priming, draining lymph node T cell proliferation in response to pigeon fragment 81-104 (*a* and *b*) or PPD (*c*) was determined in a [$^3$H]thymidine uptake assay. The results are expressed as Δ cpm. cpm values for no antigen controls ranged from 1,779 ± 304 to 3,343 ± 637.

tolerogenic) splenocytes. The results were the same (data not shown). It should be noted that B10.A(4R) splenocytes, ECDI-coupled with GAT, an antigen recognized in association with $A^k_\beta$:$A^k_\alpha$ (32), induced specific unresponsiveness to GAT in syngeneic recipients (data not shown). This control showed that antigen-coupled B10.A(4R) splenocytes could be tolerogenic when their expressed Ia molecule was involved in activation.

Finally, to rule out a role for I-J in this particular form of tolerance induction, B10.A(3R) mice ($A^b_\beta$, $A^b_\alpha$, $E^b_\beta$, $E^k_\alpha$, $I\text{-}J^b$) were injected intravenously with B10.A(5R) splenocytes ($A^b_\beta$, $A^b_\alpha$, $E^b_\beta$, $E^k_\alpha$, $I\text{-}J^k$) coupled via ECDI with a synthetic analog of moth cytochrome *c*, 86-89;93-103(93E,98A). As shown in Fig. 10 this I-J-incompatible combination induced the maximum level of unresponsiveness attainable with the syngeneic combination. In contrast, B10.A(18R) splenocytes ($A^b_\beta$, $A^b_\alpha$, $E^b_\beta$, $E^b_\alpha$, $I\text{-}J^b$), which are I-J-compatible with B10.A(3R) but do not express $E_\beta$:$E_\alpha$ molecules, failed to induce maximal unresponsiveness. The partial unresponsiveness observed in this case may be due to the existence of $A^b_\beta$:$A^b_\alpha$-restricted, 86-89;93-103(93E,98A)–specific clones in B10.A(3R) mice. Taken together, these experiments demonstrate that the induction of T cell unresponsiveness in vivo to pigeon cytochrome *c* has the same antigen and Ia molecule specificity (fragment 81-104 and $E^k_\beta$:$E^k_\alpha$) as does the induction of T cell unresponsiveness in vitro. We conclude from these experiments that this form of tolerance induction is MHC-restricted.

## Discussion

In this report we demonstrate that antigen presentation by chemically modified APC can induce unresponsiveness in vitro for T cell clones and in vivo for lymph node T cells. The induction of this unresponsiveness had the same antigen and MHC specificity as T cell activation, i. e., it depended on both the $E^k_\beta$:$E^k_\alpha$ Ia

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

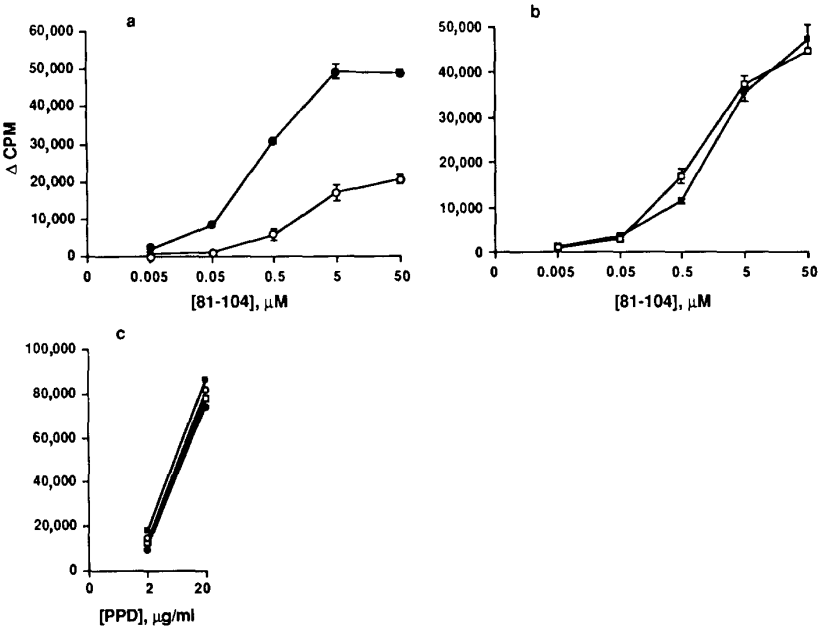


FIGURE 9. Fragment 81-104, ECDI-coupled B10.A(4R) splenocytes do not induce unresponsiveness in B10.A. B10.A mice (two per group) were injected intravenously with $5 \times 10^7$ ECDI-treated B10.A splenocytes (*a*) coupled with nothing (*filled circles*) or pigeon fragment 81-104 (*open circles*), or with $5 \times 10^7$ B10.A(4R) ECDI-treated splenocytes (*b*) coupled with nothing (*filled squares*) or fragment 81-104 (*open squares*). 1 d later, mice were primed with pigeon cytochrome *c* and 7 d later, T cell proliferation was determined as described in the legend to Fig. 8. The results are expressed as Δ cpm. cpm values for no antigen controls ranged from 657 ± 117 to 1,098 ± 103. (*a* and *b*) the response to pigeon fragment 81-104; (*c*) the response to PPD.

molecule and on residues contained within the pigeon cytochrome *c* fragment 81-104. Furthermore, the failure of moth 93-103(93E,99Q) to induce unresponsiveness in vitro demonstrated the requirement for recognition of the lysine at position 99, a residue that has been suggested (29) to contact the T cell receptor of clones specific for pigeon fragment 81-104. Our findings are consistent with a recent report (33) that demonstrated that the specificity of the induction of neonatal tolerance in vivo was identical to that of T cell activation and other reports (4, 34–37) that have demonstrated that tolerance induction in vivo and in vitro is MHC-restricted.

Previous work has shown (2, 38) that T suppressors contribute to unresponsiveness by inhibiting the function of helper and delayed-type hypersensitivity T cells. Several points suggest that the in vivo unresponsiveness examined in this paper is not mediated by suppression. T suppressors generally do not inhibit T cell proliferation (13, 38), although some examples have been reported (39, 40). Furthermore, in several instances proliferative T cells and T suppressors have been shown to recognize distinct portions of the same antigen (8). Oki and Sercarz (1) have shown that removal of a suppressor determinant destroys the

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

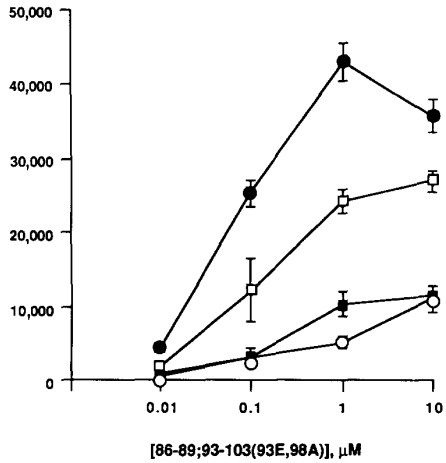


FIGURE 10. *I-J* compatibility is not required for the induction of T cell unresponsiveness in vivo. B10.A(3R) mice (two per group) were injected intravenously with $5 \times 10^7$ ECDI-treated B10.A(3R) splenocytes coupled with nothing (*filled circles*) or $5 \times 10^7$ ECDI-treated B10.A(3R) (*open circles*), B10.A(5R) (*filled squares*), or B10.A(18R) (*open squares*) splenocytes coupled with moth 86-89;93-103(93E,98A). 1 d later, mice were primed with 10 nmol of moth 86-89;93-103(93E,98A) and 7 d later, T cell proliferation was determined in a [$^3$H]thymidine uptake assay. The results are expressed as Δ cpm. cpm values for no antigen controls ranged from 524 ± 29 to 1,140 ± 735.

tolerogenicity of hen egg white lysozyme. In contrast, pigeon cytochrome *c* fragment 81-104, which contains the antigenic determinant recognized by proliferative T cells, induced unresponsiveness when associated with the $E_\beta^k$:$E_\alpha^k$ Ia molecule, suggesting a direct effect of the tolerogen on the responding T cells. However, the failure of fragment 81-104, ECDI-coupled B10.A(4R) splenocytes (I-$J^b$) to tolerize the pigeon cytochrome *c* response of B10.A or B10.A(2R) recipients (I-$J^k$) could also be attributed to the requirement for I-J compatibility between the tolerogen and suppressor T cells (2, 4). That this is not the case is indicated by experiments in which moth cytochrome *c*, ECDI-coupled B10.A(5R) splenocytes ($E_\beta^b$, $E_\alpha^k$, $I\text{-}J^k$) induced maximal moth cytochrome *c*–specific T cell unresponsiveness in I-J-incompatible B10.A(3R) recipients ($E_\beta^b$, $E_\alpha^k$, $I\text{-}J^b$), whereas moth cytochrome *c*, ECDI-coupled I-J-compatible B10.A(18R) splenocytes ($E_\beta^b$, $E_\alpha^b$, $I\text{-}J^b$) did not. Thus, I-E compatibility appears to be necessary and sufficient for this form of rapid tolerance induction.

Our observation that cloned T cells could be rendered unresponsive in vitro suggests a direct effect of antigen and ECDI-treated APC on the specific T cells. Because ECDI-treated B cells plus macrophages, essentially depleted of T cells, could induce unresponsiveness in the presence of antigen, an indirect mechanism involving suppressor T cell induction would seem to be unlikely. Furthermore, studies by Quill and Schwartz[3] using planar lipid membranes to induce a similar nonresponsive state in vitro, have demonstrated that only Ia molecules and

[3] Quill, H. and R. H. Schwartz, 1986. Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. Submitted for publication.

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

antigen are required for this effect. The identical specificity of T cell unresponsiveness induced in vitro and in vivo with that required to stimulate T cell proliferation in vitro suggests that the same receptor occupancy event occurs in each instance. These results strongly argue that a functional clonal deletion mechanism underlies the in vivo unresponsiveness we have observed in our system. Thus, it should be possible for the first time to dissect out the biochemical basis of T cell unresponsiveness using the in vitro correlate of an in vivo model system.

One model for functional clonal deletion involves the blockade of T cell receptors by antigen/MHC molecules (5, 30). Our results suggest that T cell receptor blockade or modulation does not account for T cell inactivation since the in vitro–induced unresponsiveness lasted for at least 8 d in the absence of tolerogen. Others have demonstrated (41) significant cell surface reexpression of T cell receptors modulated by mAbs only 48 h after antibody treatment. Furthermore, Quill and Schwartz[3] have shown that T cell clones express normal levels of T cell receptor molecules at times when the cells are completely unresponsive. However, it is still formally possible that antigen/Ia molecules derived from the tolerogen remain associated with the T cell clones for long periods of time without interfering with the binding of an anti-$V_\beta$ mAb.

We would propose that the T cells recognize antigen in association with Ia molecules on the surface of ECDI-treated APC in a manner similar to that which occurs on normal APC. However, the ECDI treatment appears to impair an additional APC signal necessary to induce IL-2 production and T cell proliferation. This interpretation is consistent with the recent results of Weiss et al. (42), which demonstrate that certain T cells require T cell receptor perturbation plus additional signals to produce IL-2. Our results extend this by suggesting that incomplete signaling in combination with T cell receptor occupancy by antigen/Ia molecules results in an unresponsive state. This state appears not to involve inhibition of the IL-2-R pathway, as T cell clones unable to respond to antigen/Ia molecule restimulation responded normally to exogenous IL-2. In addition, this unresponsive state is induced in the absence of T cell proliferation, distinguishing it from unresponsiveness induced by exposure of T cells to high doses of IL-2 (43). Thus, the model first proposed for B cells by Bretscher and Cohn (44) and later modified by others (3, 6), in which receptor occupancy in the absence of other signals results in tolerance induction, could be applied to T cells. If so, T and B cells would use similar mechanisms of tolerance induction. In this regard, it should be noted that the in vitro T cell unresponsiveness reported here and the in vitro B cell unresponsiveness reported by others (45) have similar time courses of induction.

Preliminary evidence from our laboratory suggests that the T cells are partially activated after incubation with antigen and ECDI-treated APC and that this partial activation renders the T cell incapable of producing IL-2 for at least 8 d in vitro. This interpretation differs from previous conclusions on the cellular basis for antigen-coupled, cell-induced unresponsiveness. It has been proposed by others (3, 4) that the tolerogenicity of antigen- or hapten-coupled cells is related to the high antigen density on these cells and the intravenous route of their administration. Our results suggest that a crucial function of ECDI is not

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

only to couple antigen to the splenocytes but also to inactivate the APC accessory signal(s) necessary to fully activate T cells.

Lamb et al. (11) have also reported the in vitro induction of unresponsiveness after incubation of human T cell clones with antigen fragments in the absence of APC. Since subsequent studies (12) showed that the induction of unresponsiveness could be blocked with anti–class II MHC antibodies, it is possible that the class II MHC–positive human T cells were presenting antigen to each other. Therefore, in both murine and human systems, nonmitogenic antigen presentation in vitro appears to result in unresponsiveness. A prediction of this type of model would be that other cells that express surface class II MHC molecules but do not support T cell proliferation, e. g., UV-irradiated splenocytes (46), gamma-irradiated (3,000 rad) resting B cells (47), neonatal splenocytes (48), and possibly thymic epithelial cells (49), might induce unresponsiveness. Recently published experiments (50) have shown that even certain nonstimulatory, anti-T cell receptor mAbs induced a state of unresponsiveness, further supporting the idea that receptor occupancy under nonmitogenic conditions can result in T cell inactivation.

In summary, we have shown that nonmitogenic, antigen presentation by ECDI-treated APC induces inhibition of T cell clones in vitro and in vivo, suggesting that functional clonal deletion is one mechanism of tolerance induction. Future experiments will be aimed at identifying the biochemical events resulting in unresponsiveness, and the nature of the ECDI-sensitive molecule(s) on the APC. Finally, the possible application of this procedure to inducing specific unresponsiveness in ongoing autoimmune disease as well as before organ transplantation is currently under investigation.

## Summary

We investigated the antigen specificity and presentation requirements for inactivation of T lymphocytes in vitro and in vivo. In vitro studies revealed that splenocytes treated with the crosslinker 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (ECDI) and soluble antigen fragments failed to stimulate significant proliferation by normal pigeon cytochrome *c*–specific T cell clones, suggesting that the chemical treatment inactivated full antigen presentation function. However, T cell clones exposed to ECDI-treated splenocytes and antigen in vitro were rendered unresponsive for at least 8 d to subsequent antigen stimulation with normal presenting cells. As predicted by the in vitro results, specific T cell unresponsiveness was also induced in vivo in B10.A mice injected intravenously with B10.A, but not B10.A(4R), splenocytes coupled with pigeon cytochrome *c* via ECDI. The antigen and MHC specificity of the induction of this T cell unresponsiveness in vitro and in vivo was identical to that required for T cell activation. These results suggest that nonmitogenic T cell recognition of antigen/MHC on ECDI-modified APCs results in the functional inactivation of T cell clones.

We thank Ms. S. Starnes and Ms. J. Eccard for preparation of this manuscript; Drs. J. Ashwell, D. Cohen, B. Fox, R. Germain, K. McCoy, D. Pardoll, H. Quill, and A. Singer for critical reading of the manuscript; and Ms. Chuan Chen for expert technical assistance.

*Received for publication 16 September 1986.*

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

## References


1. Oki, A., and E. Sercarz. 1985. T cell tolerance studied at the level of antigenic determinants. I. Latent reactivity to lysozyme peptides that lack suppressogenic epitopes can be revealed in lysozyme-tolerant mice. *J. Exp. Med.* 161:897.
2. Germain, R. N., and B. Benacerraf. 1981. A single major pathway of T lymphocyte interactions in antigen-specific immune suppression. *Scand. J. Immunol.* 13:1.
3. Claman, H. N., S. D. Miller, P. J. Conlon, and J. W. Moorhead. 1980. Control of experimental contact sensitivity. *Adv. Immunol.* 30:121.
4. Monroe, J. G., A. Lowy, R. D. Granstein, and M. I. Greene. 1984. Studies of immune responsiveness and unresponsiveness to the *p*-azobenzenearsonate (ABA) hapten. *Immunol. Rev.* 80:103.
5. Borel, Y. 1980. Haptens bound to self IgG induce immunologic tolerance, while when coupled to syngeneic spleen cells they induce immune suppression. *Immunol. Rev.* 50:71.
6. Nossal, G. J. V. 1983. Cellular mechanisms of immunologic tolerance. *Annu. Rev. Immunol.* 1:33.
7. Taniguchi, M., and J. F. A. P. Miller. 1977. Enrichment of specific suppressor T cells and characterization of their surface markers. *J. Exp. Med.* 146:1450.
8. Goodman, J. W., and E. E. Sercarz. 1983. The complexity of structures involved in T-cell activation. *Annu. Rev. Immunol.* 1:465.
9. Asano, Y., and R. J. Hodes. 1983. T cell regulation of B cell activation. Cloned Lyt $1^+2^-$ T suppressor cells inhibit the major histocompatibility complex-restricted interaction of T helpers with B cells and/or accessory cells. *J. Exp. Med.* 158:1178.
10. Jenkins, M. K., and S. D. Miller. 1985. Immunoregulatory pathways in adult responder mice. III. Establishment of a GAT-specific suppressor T cell clone from GAT-tolerant responders which afferently regulates DTH responses. *J. Mol. Cell. Immunol.* 2:1.
11. Lamb, J. R., B. J. Skidmore, N. Green, J. M. Chiller, and M. Feldmann. 1983. Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. *J. Exp. Med.* 157:1434.
12. Lamb, J. R., and M. Feldmann. 1984. Essential requirement for major histocompatibility complex recognition in T-cell tolerance induction. *Nature (Lond.).* 308:72.
13. Miller, S. D., R. P. Wetzig, and H. N. Claman. 1979. The induction of cell-mediated immunity and tolerance with protein antigens coupled to syngeneic lymphoid cells. *J. Exp. Med.* 149:758.
14. Brautigen, D. L., S. Ferguson-Miller, and E. Margoliash. 1978. Mitochondrial cytochrome *c*: preparation and activity of native and chemically modified cytochromes *c*. *Methods Enzymol.* 53:128.
15. Corradin, G., and H. A. Harbury. 1970. Cleavage of cytochrome *c* with cyanogen bromide. *Biochim. Biophys. Acta.* 221:489.
16. Schwartz, R. H., B. S. Fox, E. Fraga, C. Chen, and B. Singh. 1985. The T lymphocyte response to cytochrome *c*. V. Determination of the minimal peptide size required for stimulation of T cell clones and assessment of the contribution of each residue beyond this size to antigenic potency. *J. Immunol.* 135:2598.
17. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* 3:465.
18. Ozato, K., N. Mayer, and D. H. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* 124:533.
19. Oi, V. T., P. P. Jones, J. W. Goding, and L. A. Herzenberg. 1978. Properties of monoclonal antibodies to mouse Ig allotypes, H-2, and Ia antigens. *Curr. Top. Microbiol. Immunol.* 81:115.


Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

20. McGrath, M. S., E. Pillemer, and I. L. Weissman. 1980. Murine leukemogenesis: monoclonal antibodies to T cell determinants arrest T-lymphoma cell proliferation. *Nature (Lond.)*. 285:259.
21. Ledbetter, J. A., and L. A. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* 47:63.
22. Lanier, L. L., G. A. Gutman, D. E. Lewis, S. T. Griswold, and N. L. Warner. 1982. Monoclonal antibodies against rat immunoglobulin kappa chains. *Hybridoma.* 1:125.
23. Matis, L. A., D. L. Longo, S. M. Hedrick, C. Hannum, E. Margoliash, and R. H. Schwartz. 1983. Clonal analysis of the major histocompatibility complex-restriction and the fine specificity of antigen recognition in the T cell proliferative response to cytochrome *c*. *J. Immunol.* 130:1527.
24. Ashwell, J. D., B. S. Fox, and R. H. Schwartz. 1986. Functional analysis of the interaction of the antigen-specific T cell receptor with its ligands. *J. Immunol.* 136:757.
25. Suzuki, G., and R. H. Schwartz. 1986. The pigeon cytochrome *c*-specific T cell response of low responder mice. I. Identification of antigenic determinants on fragment 1-65. *J. Immunol.* 136:230.
26. Kimoto, M., and C. G. Fathman. 1981. Antigen-reactive T cell clones. I. Transcomplementing hybrid I-A region gene products function effectively in antigen presentation. *J. Exp. Med.* 152:759.
27. Ashwell, J. D., C. Chen, and R. H. Schwartz. 1986. High frequency and nonrandom distribution of alloreactivity in T cell clones selected for recognition of foreign antigen in association with self class II molecules. *J. Immunol.* 136:389.
28. Bhattacharya, A., M. E. Dorf, and T. A. Springer. 1981. A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: evidence for I region gene duplication. *J. Immunol.* 127:2488.
29. Schwartz, R. H. 1985. T-Lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Annu. Rev. Immunol.* 3:237.
29a. Quill, H., L. Carlson, B. S. Fox, J. Weinstein, and K. H. Schwartz. 1986. Optimization of antigen presentation to T cell hybridomas by purified Ia molecules in planar membranes. Ia molecule polymorphism determines the antigenic fine specificity of the response to cytochrome *c* peptides. *J. Immunol. Methods.* In press.
30. Miller, S. D., M. S. Sy, and H. N. Claman. 1977. The induction of hapten-specific T cell tolerance using hapten-modified lymphoid cells. II. Relative roles of suppressor T cells and clone inhibition in the tolerant state. *Eur. J. Immunol.* 7:165.
31. Solinger, A. M., M. E. Ultee, E. Margoliash, and R. H. Schwartz. 1979. T lymphocyte response to cytochrome *c*. I. Demonstration of a T-cell heteroclitic proliferative response and identification of a topographic antigenic determinant on pigeon cytochrome *c* whose immune recognition requires two complementing major histocompatibility complex-linked immune response genes. *J. Exp. Med.* 150:830.
32. Schwartz, R. H., and W. E. Paul. 1976. T-Lymphocyte-enriched murine peritoneal exudate cells. II. Genetic control of antigen-induced T-lymphocyte proliferation. *J. Exp. Med.* 143:529.
33. Gammon, G., K. Dunn, N. Shastri, A. Oki, S. Wilbur, and E. E. Sercarz. 1986. Neonatal T-cell tolerance to minimal immunogenic peptides is caused by clonal inactivation. *Nature (Lond.)*. 319:413.
34. Groves, E. S., and A. Singer. 1983. Role of the H-2 complex in the induction of T cell tolerance to self minor histocompatibility antigens. *J. Exp. Med.* 158:1483.
35. Matzinger, P., R. Zamoyska, and H. Waldmann. 1984. Self-tolerance is H-2 restricted. *Nature (Lond.)*. 308:739.
36. Rammensee, H., and M. J. Bevan. 1984. Evidence from *in vitro* studies that tolerance to self antigens is MHC-restricted. *Nature (Lond.)*. 308:741.

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024

37. Conlon, P. J., J. W. Moorhead, and H. N. Claman. 1979. Efficient induction of immediate tolerance to contact sensitivity by hapten-modified spleen cells requires $Ia^+$ cells compatible with the recipient. *Nature (Lond.)*. 278:257.
38. Jenkins, M. K., C. Waltenbaugh, and S. D. Miller. 1985. Immunoregulatory pathways in adult responder mice. II. Regulation of delayed-type hypersensitivity responses by GAT-specific suppressor factors present in GAT-tolerant adult responder mice. *J. Immunol.* 134:114.
39. Kapp, J. A., B. A. Araneo, and B. L. Clevinger. 1980. Suppression of antibody and T cell proliferative responses to L-glutamic $acid^{60}$-L-$alanine^{30}$-L-$tyrosine^{10}$ by a specific monoclonal T cell factor. *J. Exp. Med.* 152:235.
40. Baxevanis, C. N., N. Ishii, Z. A. Nagy, and J. Klein. 1982. H-2-controlled suppression of T cell response to lactate dehydrogenase B. Characterization of the lactate dehydrogenase B suppressor pathway. *J. Exp. Med.* 156:822.
41. Reinherz, E. L., S. Meuer, K. A. Fitzgerald, R. E. Hussey, H. Levine, and S. F. Schlossman. 1982. Antigen recognition by human T lymphocytes is linked to surface expression of the T3 molecular complex. *Cell.* 30:735.
42. Weiss, A., R. Wiskocil, and J. Stobo. 1984. The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL-2 production reflects events occurring at a pre-translational level. *J. Immunol.* 133:1.
43. Otten, G., D. B. Wilde, M. B. Prystowsky, J. S. Olshan, H. Rabin, L. E. Henderson, and F. W. Fitch. 1986. Cloned helper T cells exposed to IL-2 become unresponsive to antigen and concanavalin A but not to calcium ionophore and phorbol ester. *Eur. J. Immunol.* 16:217.
44. Bretscher, P., and M. Cohn. 1970. A theory of self-nonself discrimination: paralysis and induction involve the recognition of one and two determinants on an antigen, respectively. *Science (Wash. DC)*. 169:1042.
45. Nossal, G. J. V., and B. L. Pike. 1978. Mechanisms of clonal abortion tolerogenesis. I. Response of immature, hapten-specific B lymphocytes. *J. Exp. Med.* 148:1161.
46. Letvin, N. L., I. Fox, M. I. Greene, B. Benacerraf, and R. N. Germain. 1980. Immunologic effects of whole body ultraviolet (UV) irradiation. II. Defect in splenic adherent cell antigen presentation for stimulation of T cell proliferation. *J. Immunol.* 125:1402.
47. Ashwell, J. D., A. L. DeFranco, W. E. Paul, and R. H. Schwartz. 1984. Antigen presentation by resting B cells. Radiosensitivity of the antigen-presentation function and two distinct pathways of T cell activation. *J. Exp. Med.* 159:881.
48. Lu, C. Y., D. I. Beller, and E. R. Unanue. 1980. A defect in the antigen presenting function of macrophages from neonatal mice. *Nature (Lond.)*. 282:327.
49. Kyewski, B. A., C. G. Fathman, and R. V. Rouse. 1986. Intrathymic presentation of circulating non-MHC antigens by medullary dendritic cells. An antigen-dependent microenvironment for T cell differentiation. *J. Exp. Med.* 163:231.
50. Tomonari, K. 1985. T cell receptor expressed on an auto-reactive T-cell clone, clone 4. I. Induction of various T-receptor functions by anti-T idiotypic antibodies. *Cell. Immunol.* 96:147.

Downloaded from http://rupress.org/jem/article-pdf/165/2/302/1667752/302.pdf by guest on 09 March 2024