

Early Signaling Defects in Human T Cells Anergized by T Cell Presentation of Autoantigen

By Janine M. LaSalle, Paul J. Tolentino, Gordon J. Freeman,*
Lee M. Nadler,* and David A. Hafler

*From the Center for Neurologic Diseases, Brigham and Women's Hospital; *Division of Tumor Immunology, Dana Farber Cancer Institute; and Harvard Medical School, Boston, Massachusetts 02115*

Summary

Major histocompatibility complex class II-positive human T cell clones are nontraditional antigen-presenting cells (APCs) that are able to simultaneously present and respond to peptide or degraded antigen, but are unable to process intact protein. Although T cell presentation of peptide antigen resulted in a primary proliferative response, T cells that had been previously stimulated by T cells presenting antigen were completely unresponsive to antigen but not to interleukin 2 (IL-2). In contrast, peptide antigen presented by B cells or DR2⁺ L cell transfectants resulted in T cell activation and responsiveness to restimulation. The anergy induced by T cell presentation of peptide could not be prevented by the addition of either autologous or allogeneic B cells or B7⁺DR2⁺ L cell transfectants, suggesting that the induction of anergy could occur in the presence of costimulation. T cell anergy was induced within 24 h of T cell presentation of antigen and was long lasting. Anergized T cells expressed normal levels of T cell receptor/CD3 but were defective in their ability to release [Ca²⁺]_i to both αCD3 and APCs. Moreover, anergized T cells did not proliferate to αCD2 monoclonal antibodies or αCD3 plus phorbol myristate acetate (PMA), nor did they synthesize IL-2, IL-4, or interferon γ mRNA in response to either peptide or peptide plus PMA. In contrast, ionomycin plus PMA induced both normal proliferative responses and synthesis of cytokine mRNA, suggesting that the signaling defect in anergized cells occurs before protein kinase C activation and [Ca²⁺]_i release.

Despite the appeal of the discoveries in recent years showing that autoreactive T cells are deleted in the thymus (1–4), such a mechanism cannot completely account for the ability of the immune system to avoid reactivity to self. Autoreactive T cells can be isolated from the periphery from individuals both with and without incidence of tissue-specific autoimmune disease, suggesting that self-reactive T cells may exist without pathologic consequence in the periphery (5). One could argue that in most instances autoreactive T cells in the periphery of normal individuals would not normally be exposed to self-antigen presented in the context of the appropriate APC and would therefore be harmless. However, a more cautiously evolved immune system would have evolved several “fail-safe” mechanisms to distinguish self from foreign in order to prevent autoimmunity.

There have been many studies showing the phenomena of peripheral tolerance, but as yet the molecular mechanisms of these phenomena are unknown. Several systems have shown antigen-specific induction of unresponsiveness by the presence of antigen in an atypical form (6–8). High-dose tolerance, in which T cells exposed to a supraoptimum concentration of antigen are less responsive than at lower concentrations,

has been speculated to be due to repeated antigenic stimulation of the T cells by Ia-peptide complexes (9). Lamb et al. (10) developed a system of unresponsiveness similar to high-dose tolerance in which human T cell clones pretreated with their cognate peptide in the absence of APCs were rendered unresponsive to antigen upon subsequent stimulation. Both the appropriate MHC molecule and the appropriate peptide antigen were required to create this state of antigen-induced antigen-specific tolerance (11).

Antigen presented by splenocytes that have been chemically crosslinked by 1-ethyl-3-(dimethylaminopropyl)carbodiimide (ECDI) can also induce a state of T cell unresponsiveness to that antigen in vivo (12). Schwartz and colleagues (6–8) have investigated this phenomenon extensively in vitro and have hypothesized that antigen unresponsiveness, or anergy, results from inappropriate antigen presentation in which a T cell received a signal through its TCR/CD3 complex without receiving a second costimulation. Support for this model has come from other in vitro systems that use immobilized α CD3 to anergize T cell clones (13). The ligation of CD28 on T cells by αCD28 mAb or its natural ligand, B7, expressed on accessory cells appears to provide costimu-

lation for IL-2 production by T cells (14–16). This pathway has been speculated to be involved in the prevention of anergy by traditional APCs, although such a mechanism has not been clearly demonstrated.

Evidence for peripheral tolerance has also been observed in transgenic mice in which foreign MHC is expressed only in tissue-specific cells in the periphery (17–19). In these mice, T cells reactive with this antigen are not deleted but are unresponsive to stimulation through the TCR. A similar TCR unresponsiveness has been seen in peripheral T cells from transgenic mice and bone marrow chimeric mice, which have incomplete deletion of autoreactive T cells (20, 21). In an example of a potentially different mechanism of peripheral tolerance in vivo, mature autoreactive T cells are first expanded and then eliminated in the periphery after exposure to antigen (19). Although studies can be performed to characterize the state of anergy in terms of signaling pathways on anergized T cells isolated from these in vivo situations, it is difficult to study the mechanism that induces anergy.

Here we show a system of T cell anergy in human T cells that results from stimulation by peptide antigen presented by T cells as opposed to traditional APCs (22). The addition of antigen presenting and nonpresenting accessory cells does not prevent the induction of unresponsiveness by T cells presenting antigen, suggesting that anergy results from a dominant negative signal rather than the lack of positive costimulation, as has been suggested in other systems (6–8). The block in signaling of anergized T cells appears to be before the release of $[Ca^{2+}]_i$, as anergized T cells fail to release $[Ca^{2+}]_i$ in response to antigenic stimulation, but can be stimulated to proliferate by the combination of Ca^{2+} ionophore and the phorbol ester PMA. These results may provide a mechanism to explain one model of T cell tolerance in which free peptide antigen induces tolerance in responding T cell clones.

Materials and Methods

Generation of MBP-reactive T Cell Clones. Myelin basic protein (MBP)¹-reactive T cell lines and clones were generated as described previously (5). T cell clones were generated from MBP 84-102-reactive T cell lines by limiting dilution (0.3 cells/well) in 96-well V-bottomed microtiter plates (Costar, Cambridge, MA) in the presence of 1 μ g/ml PHA.P (Wellcome Diagnostics, Beckenham, UK) and 10^5 irradiated allogeneic PBMC in media consisting of RPMI 1640 (Whittaker, Walkersville, MD), 10% pooled human AB serum (PHS) (Biocell, Carson City, CA), 4 mM glutamine (Gibco Laboratories, Grand Island, NY), 10 mM Hepes (Whittaker), 100 U/ml penicillin/streptomycin (Gibco Laboratories), 5% IL-2 (Human T stim; Collaborative Research, Bedford, MA), and 1 U/ml rIL-4 (kindly supplied by Genetics Institute, Cambridge, MA). Clones were restimulated every 8–12 d at 10^4 /well in 96-well round-bottomed microtiter plates (Costar) with alternating rounds of 40 μ M 84-102-pulsed, irradiated autologous PBMC and PHA/allogeneic PBMC. For the experiments shown here, T cell clones were restimulated from aliquots of the same freeze to prevent drift from long term culture. T cells were frozen in 90% FCS/10% DMSO in liquid nitrogen.

¹ Abbreviations used in this paper: MBP, myelin basic protein; PKC, protein kinase C.

MBP Peptide 84-102. MBP peptide 84-102, amino acid sequence DENPVVHFFKNIVTPRTPP, was synthesized by the solid phase method (Biosearch Lab, Inc., San Raphael, CA) and purified on HPLC as previously reported (5).

Cell Lines. Previously established EBV-transformed B cell lines 9010 (DR2w2, DQ1) and 9009 (DR2w21, DQ1) were grown in 10% FCS complete media then frozen as described above and thawed immediately before antigen pulse. Mouse fibroblast L cell line transfected with DR2a (kind gift of R. Sekaly, Clinical Research Institute, Montreal, Quebec, Canada) were grown in DMEM (Whittaker), 10% FCS (Whittaker), 1.6 mM xanthine (Sigma Chemical Co., St. Louis, MO), 110 μ M hypoxanthine (Sigma Chemical Co.), 18 μ M mycophenolic acid (Gibco laboratories) selection media, frozen as described above, and thawed immediately before antigen pulse. HT-2 murine IL-2-dependent T cell line (American Type Culture Collection, Rockville, MD) was grown in 10% FCS complete media with 5% IL-2 and used for IL-2 bioassay 2 d after the last feeding.

mAbs. mAbs used in these studies include CD3-specific OKT3 and 2AD2, CD2-specific T11₂ and T11₃ (kind gifts from S. Schlossman, Dana Farber Cancer Institute, Boston, MA), HLA-DR-specific L243, HLA-DQ-specific S3/4 (kind gifts from F. Bach, University of Minnesota, Minneapolis, MN), HLA-DP-specific B7/21 (kind gift from N. Flomenberg, Sloan-Kettering Cancer Center, New York, NY), CD28-reactive 9.3 (kind gift from J. Ledbetter, Oncogen, Seattle, WA), CD45RA-reactive 2H4 (S. Schlossman), CD45RO-specific UCHL-1 (Dakopatts, Glostrup, Denmark), LFA-3, and LFA-1 (kind gifts from T. Springer, Center for Blood Research, Boston, MA), and CD25 Tac (kind gift of T. Waldman, NCI, NIH, Bethesda, MD).

Proliferation Assay. T cell clone (10^5 /well) was plated in triplicate and cocultured with appropriate stimuli, designated in the figure legends, for 72 h at 37°C, 90% humidity, 5% CO₂, in 96-well flat-bottomed microtiter plates (Costar) pulsed with 2 μ Ci [³H]-TdR (2 Ci/mmol; New England Nuclear, Boston, MA) for the last 18 h of culture. APCs were prepared by pulsing B cells or L cells at 10^6 cells/ml in complete media either in the presence or absence of 40 μ M MBP 84-102 for 2 h at 37°C, washing twice with 4°C HBSS (Whittaker), followed by irradiation with 5,000 rad at 4°C. To stimulate T cells without accessory APCs, 2 μ M MBP peptide 84-102, or 10^3 U/ml rIL-2 (Hoffman-La Roche, Inc., Nutley, NJ) was added directly to the cells for the duration of the culture.

Flow Cytometric Analysis of T Cells. A 1/100 dilution of the mAb ascites in PBS/2% PHS was used to coat T cells at 10^6 /ml for 30 min. at 4°C. Cells were washed twice with 4°C staining media, then stained with 1/60 FITC-conjugated goat anti-mouse Ig (Tago Inc., Burlingame, CA) for 30 min at 4°C. Cells were washed twice as above, then fixed with 1% formaldehyde (J. T. Baker Chemical Co, Phillipsburg, NJ). Flow cytometric analysis was performed on an Epics C flow cytometer (Coulter Electronics, Hialeah, FL).

B7 Transfection. 50 μ g KpnI-linearized B7-pCDM8 construct was cotransfected with 5 μ g of PvuI-linearized POP.F into L-tk⁻ cells or into DR2⁺ L-tk⁻ cells (previously transfected with DR2) by electroporation using an electroporator (250 V and 1,600 mF; Bethesda Research Laboratories, Gaithersburg, MD). The POP.F plasmid contains the herpes simplex virus thymidine kinase gene under the control of the SV40 promoter (23). DR2⁻B7⁺ transfectants were selected by growth in hypoxanthine/aminopterin/thymidine (Sigma Chemical Co.) containing media and cloned. DR2⁺B7⁺ transfectants were selected and cloned in xanthine/

hypoxanthine/mycophenolic acid media containing 150 $\mu\text{g/ml}$ G418 sulfate (Gibco Laboratories). Clones expressing cell surface B7, as assayed by indirect immunofluorescence with anti-B7 mAb, were recloned.

Measurement of $[Ca^{+2}]$. As described previously (22), T cell clones ($0.2\text{--}1.0 \times 10^7/\text{ml}$) were loaded with 2 $\mu\text{g/ml}$ Indo-1 (Sigma Chemical Co.) in culture media for 45 min at 37°C. Indo-loaded cells were diluted 1/10 with media and kept at 4°C until 1 min before flow cytometric analysis (Coulter Electronics). Stimulator APCs were pulsed with or without 40 μM 84-102, washed twice, and resuspended in media at 4°C. Unstimulated Indo-loaded T cells were analyzed for 30 s before addition of stimulus. In the case of cellular stimulators, stimulator APCs plus loaded responders were analyzed for 30 s, then centrifuged for 1 min at 1,500 rpm to establish cell-cell contact, then resuspended and analyzed for response. Ionomycin (100 $\mu\text{g/ml}$) (Sigma Chemical Co.) was used as a positive control for Indo-1 loading.

Detection of Cytokine mRNA by Northern Analysis. T cell clone Ob.1A12.8 ($10^5/\text{well}$ in 96-well round-bottomed microtiter plates in IL-2/IL-4-supplemented media) was grown in the presence or

absence of 2 μM 84-102 for 48 h. Cells were washed, and resuspended in complete media at $2 \times 10^6/\text{ml}$ in the presence or absence of either 10 ng/ml PMA and 1 $\mu\text{g/ml}$ ionomycin, 2 μM 84-102, or 2 μM 84-102 + 10 ng/ml PMA for 4 h at 37°C. Total cellular RNA was extracted using the RNAzol B method (TM Cinna Scientific, Friendswood, TX). 10 μg of total cellular RNA was fractionated by formaldehyde gel electrophoresis using 1.2% SeaKem ME agarose (FMC Bioproducts, Rockland, ME) and 2.2 M formaldehyde. After fractionation, RNA was blotted onto Nytran membranes (Schleicher & Schuell, Inc., Keene, NH) with a $10 \times$ SSC solution by capillary transfer overnight. Membranes were baked in a vacuum oven at 80°C for 2 h. For hybridization of cytokine probes, membranes were prewashed in $0.5 \times$ SSC and 5% SDS at 65°C for 2 h, then prehybridized in 50% formamide, $5 \times$ SSC and 0.5% SDS, $1 \times$ Denhardt's solution, 10% dextran sulfate, and 100 $\mu\text{g/ml}$ salmon sperm DNA (Sigma Chemical Co.) at 42°C for 1–2 h. Probes for IL-2, IL-4, and IFN- γ (described previously in reference 24) were labeled to a specific activity of $>10^8$ cpm/ μg by random primer labeling method (Boehringer Mannheim, Mannheim, Germany) and added to fresh hybridization buffer. Hybrid-

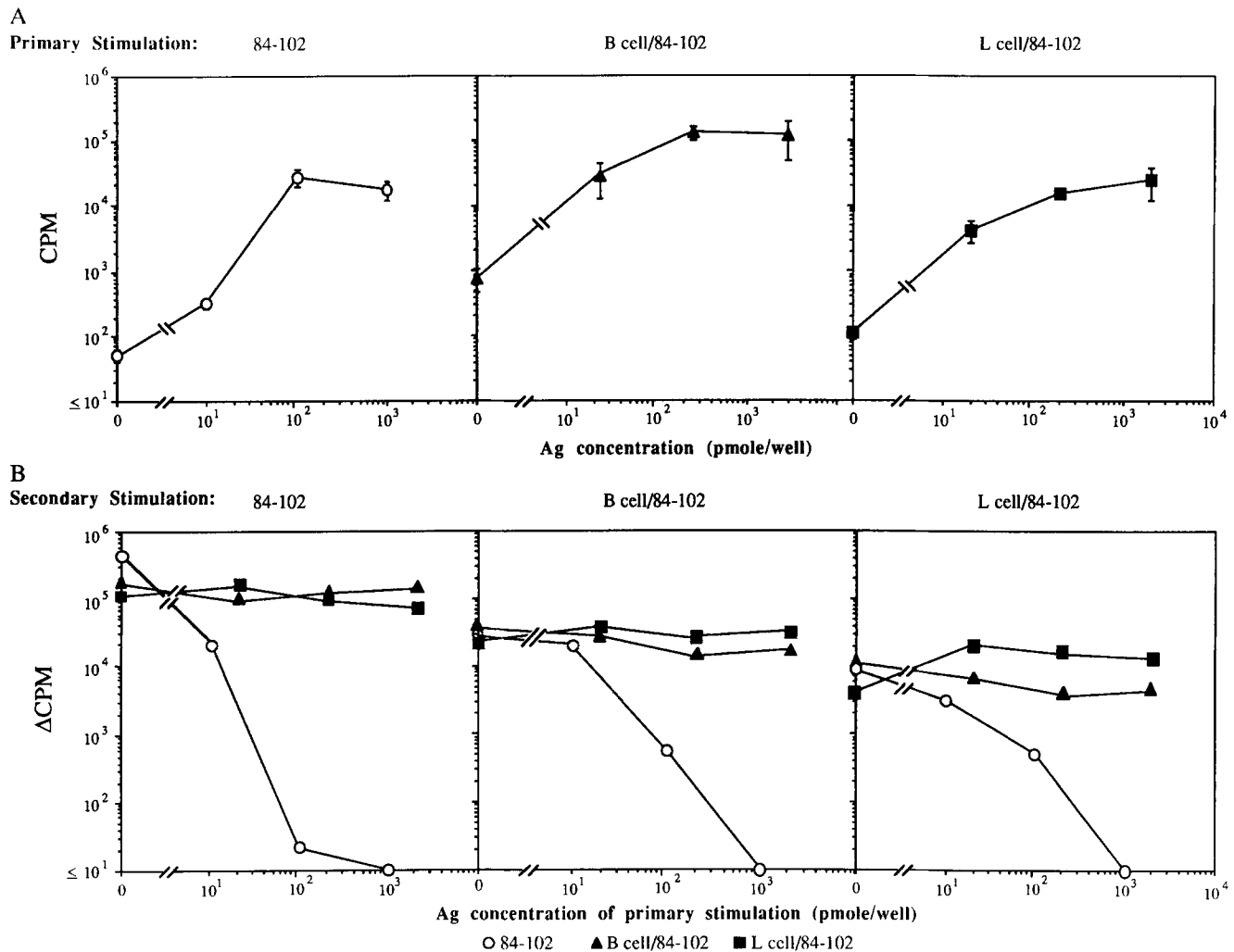


Figure 1. T cells previously stimulated with free-peptide antigen are unresponsive to antigenic stimulation. T cell clone Ob.1A12.8 was stimulated either directly with MBP peptide 84-102 or with a DR2⁺ B cell line (9010) or L cell transfectant pulsed with 84-102 for 2 h at 37°C and assayed for proliferation. Final concentration of peptide in wells of primary stimulation is indicated. As seen in *A*, all three stimuli resulted in equivalent T cell proliferation. 7 d later, T cells were washed and reassayed for response to 84-102 (5 $\mu\text{g/ml}$) or B cells or L cells pulsed with 84-102 (100 $\mu\text{g/ml}$). As shown in *B*, T cells originally stimulated with high concentrations of 84-102 were unresponsive to any secondary antigenic stimulation.

zations were performed at 42°C for 20 hrs. Filters were washed in 0.2× SSC/0.1% SDS twice for 10 min at 24°C, then twice for 30 min at 50°C. Autoradiography was performed at -70°C for 1-5 d.

Results

T Cells Previously Stimulated with Peptide Antigen Are Unresponsive to Antigen. We have previously shown that MBP-reactive T cell clones proliferate in response to peptide antigen in the absence of traditional APCs by presenting peptide antigen on their own MHC class II molecules to autologous T cell clones (22). We then examined whether T cell presentation of antigen involved different signaling events in responding T cell clones as compared with traditional APCs. As shown in Fig. 1, T cell clone Ob.1A12.8 responded as well to MBP peptide 84-102 added directly to the culture as compared with peptide bound to pulsed DR2⁺ B cell line or DR2-transfected L cells in a primary proliferation assay (Fig. 1 A). However, T cells originally stimulated by free MBP 84-102 peptide antigen were unresponsive to antigen stimulation in any form when assayed 1 wk later (Fig. 1 B, open circles), while T cells stimulated in primary cultures by peptide-pulsed B cells or DR2⁺ L cells responded normally to secondary stimulation (Fig. 1 B, filled symbols). The degree of unresponsiveness in the secondary stimulation was inversely proportional to the antigen concentration and the proliferation induced in the primary stimulation.

We have found MBP peptide induced unresponsiveness in five different T cell clones from two different individuals reactive against two different MBP peptide epitopes. Only the specific peptide epitope induces T cell unresponsiveness (data not shown).

The Addition of Costimulatory Cells Does Not Reverse Unresponsiveness. We hypothesized that the induction of anergy was due to a negative signal rather than the lack of a costimulatory signal on T cell APCs since L cells (which presumably have no human costimulator molecules) did not induce anergy. However, L cells have been recently found to express murine B7 (G. Freeman, manuscript in preparation) and murine B7 has been shown to costimulate human T cells (25). To directly test the costimulatory effect of L cells on the induction of unresponsiveness, we added DR2-transfected L cells to the culture of the T cell clone with unbound peptide antigen. To directly test the role of human B7 as a costimulator molecule, the human B7 gene was transfected into DR2⁺ and DR2⁻ L cells. As shown in Table 1, T cells cultured for a week in 84-102 were not responsive to secondary antigenic stimulation, even when L cells expressing human B7 or coexpressing B7 and DR2 were added to the culture.

To test if the unresponsiveness induced by peptide antigen was reversible by costimulator molecules other than B7, we added irradiated B7⁺ B cells that were either the appropriate (9010) or inappropriate (9009) HLA-DR for antigen presentation to the culture of T cell clone with MBP 84-102. Fig. 2 demonstrates that neither B cell line was able to prevent the induction of anergy caused by T cells responding to free peptide. However, as shown in Fig. 1, stimulation with MHC-

Table 1. Anergy Occurs in the Presence of B7 Accessory Cells

Primary stimulation			Secondary stimulation	
DR2a	B7	84-102	84-102	cpm ± SEM
+	-	No L Cell	-	192 ± 16
		-	+	32,471 ± 1,264
		+	-	303 ± 81
		+	+	344 ± 199
		-	-	347 ± 202
		-	+	14,143 ± 1,456
	+	-	-	399 ± 89
		-	+	291 ± 74
		+	-	308 ± 133
		+	+	16,354 ± 3,331
		-	-	365 ± 76
		-	+	268 ± 71
-	+	-	-	278 ± 99
		-	+	18,221 ± 7,684
		+	-	332 ± 247
	-	-	+	224 ± 89
		+	-	
		+	+	

T cell clone Ob.2F3.2 was cultured for 7 d in the presence or absence of 2 μM 84-102 with or without the addition of 10⁴/well L cells (irradiated at 5,000 rad) with expression of DR2- and/or B7-transfected molecules. L cell lines with and without expression of DR2 were transfected with B7. Mean fluorescence intensity was 77.2 (mouse Ig-PE) and 215.6 (B7-PE) for the DR2⁺B7⁺ line and 56.8 (mouse Ig-PE), and 158.6 (B7-PE) for the DR2⁻B7⁺ line. After the primary culture, T cells were washed and assayed for proliferation to 84-102. Anergic responses are highlighted in bold.

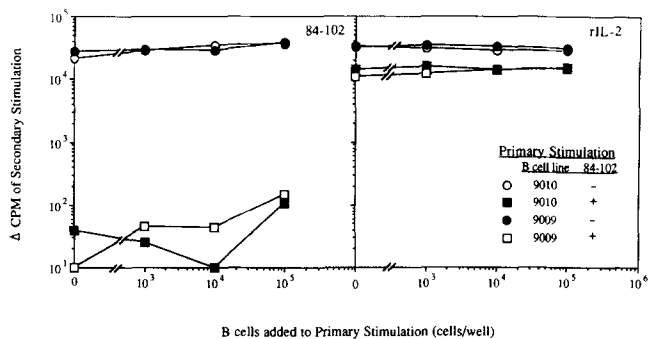


Figure 2. T cell unresponsiveness can not be prevented by the addition of B cells. T cell clone Ob.1A12.8 was cultured for 7 d of primary stimulation either alone or in the presence of 84-102 with the addition of either MHC class II-matched (9010) or -mismatched (9009) irradiated transfected B cells at the numbers indicated, then washed and assayed for proliferation to either MBP 84-102 peptide (5 μg/ml) or rIL-2 (10³ U/ml). T cells grown in the absence of peptide (circles) were fully able to proliferate to the secondary antigenic stimulation, while those grown with peptide (squares) were antigen unresponsive regardless of the addition of B cells. Proliferation to IL-2 was equivalent in all cell populations.

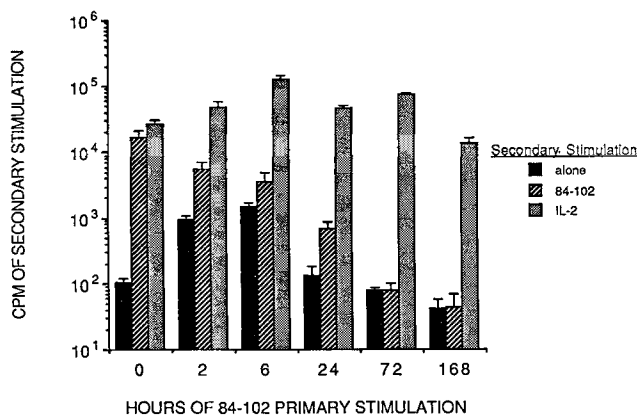


Figure 3. Kinetics of T cell anergy. T cell clone Ob.1A12.8 was cultured for 0–168 hours in 5 $\mu\text{g}/\text{ml}$ 84-102, washed, and assayed for proliferation to 84-102 or IL-2. Error bars represent SEM of cpm from triplicate cultures. Data from two consistent but separate experiments have been pooled to represent all time points.

matched B cell line 9010 that had been pulsed with peptide antigen and washed before culture did not lead to the unresponsiveness of the T cell clone. Although the T cell clone lost antigen responsiveness after exposure to peptide antigen, the response to IL-2 was unchanged, indicating that the unresponsiveness was not due to cell death.

Kinetics of Anergy Induction. To determine the kinetics of anergy induction, secondary T cell response to MBP 84-102 peptide and IL-2 was assayed after a primary stimulation with peptide from 2 to 168 h. A >10-fold reduction in the response to antigen was induced within 24 h of MBP 84-102 peptide culture, by which time the background proliferation (alone) had returned to that of resting cells. This unresponsiveness was even further enhanced to >100-fold reduction of the response over 4 d and was maintained for the duration of the experiment, 7 d (Fig. 3).

Unresponsive T Cells Continue to Express CD3. The un-

responsiveness of peptide-energized T cell clones could have been secondary to a loss of CD3/TCR cell surface expression. This was not the case, as T cells rendered unresponsive by culture with peptide antigen had an equivalent CD3 surface expression as compared with nonenergized T cell clones (Fig. 4).

Response of Anergized T Cell Clone to Nonantigenic Stimuli. To more precisely define which activation pathways are defective in energized T cell clones, T cells energized by peptide culture were treated with reagents that either mimic or bypass cell surface antigenic stimulation (Fig. 5). Anergized T cells failed to proliferate in response to either the combination of αCD3 and PMA or the αCD2 mitogenic mAbs T11₂ and T11₃. In contrast, energized T cells responded normally to the combination of PMA and ionomycin, which bypasses the need for transmembrane signaling (26), and to rIL-2, which stimulates proliferation through a separate pathway (27).

Anergized T Cells Are Defective in Their Ability to Release Calcium after Antigenic Stimulation. As we have previously shown that MBP-reactive T cell clones release $[\text{Ca}^{2+}]_i$ in response to peptide presented by antigen-pulsed T cells or B cells (22), we tested the ability of peptide-energized T cells to respond in this assay. Anergized T cells have a marked reduction in their release of $[\text{Ca}^{2+}]_i$ in response to either TCR crosslinking with αCD3 or peptide antigen presented by B cells (Fig. 6). In contrast, the response to ionomycin in energized T cell clones is equivalent to that of the nonenergized T cell clone.

Anergized T Cells Fail to Produce Cytokines in Response to Antigen. T cell clones either cultured alone or energized with MBP peptide 84-102 for 48 h in a primary culture were stimulated in a secondary culture with either peptide, peptide + PMA, or PMA + ionomycin. mRNA was extracted after 4 h and the relative quantity of IL-2, IL-4, and IFN- γ examined by Northern blotting. Before the induction of anergy, T cell clone Ob.1A12.8 synthesized IL-2, IL-4, and IFN- γ

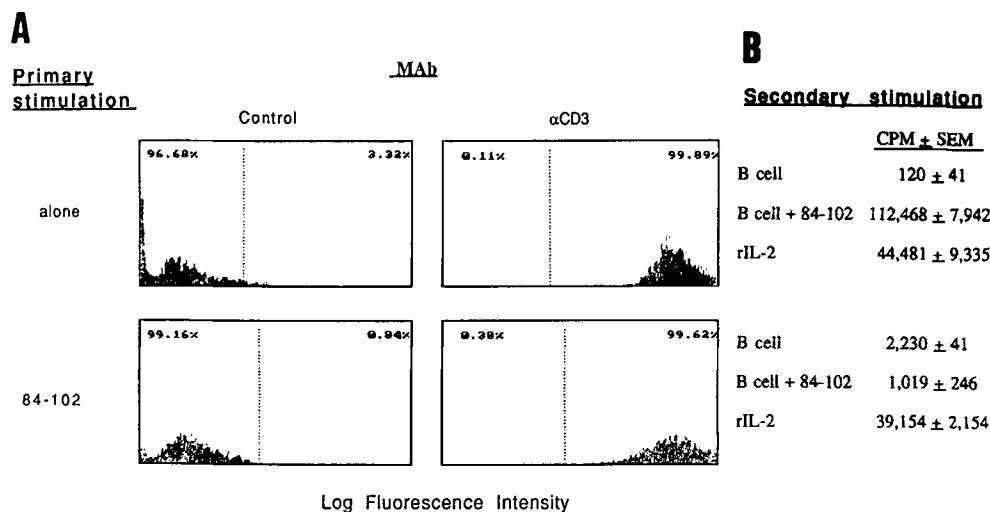


Figure 4. Anergized T cells continue to express CD3. T cell clone Ob.1A12.8 was cultured in the presence or absence of 5 $\mu\text{g}/\text{ml}$ 84-102 for 4 d, washed, and analyzed for both cell surface phenotype and proliferative response. Cells were stained with control mAb M α Ig-FITC or OKT3-FITC (Coulter) for 30 min at 4°C, washed twice, then fixed in 1% formalin and analyzed by flow cytometry. T cells from the same primary culture were assayed for proliferation to B cells (9010) pulsed with or without 84-102, or rIL-2.

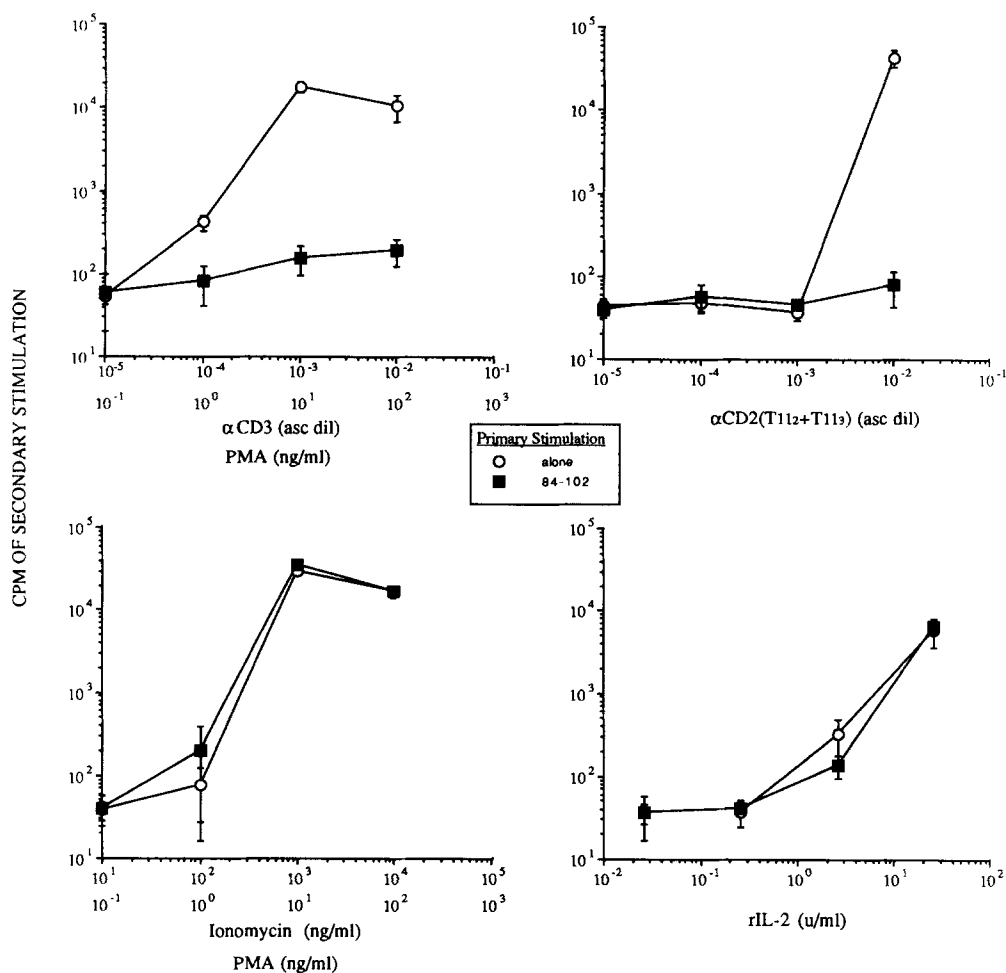


Figure 5. Activation of anergized T cells by various stimuli. T cell clone Ob.1A12.8 cultured in the presence or absence of 84-102 for 7 d was washed and assayed for proliferation to logarithmic titrations of αCD3 + PMA, T112 + T113, PMA + ionomycin, and rIL-2. Final concentration or ascites dilution of each reagent is expressed on the x-axis. PMA concentrations are listed below concentrations of αCD3 or ionomycin.

mRNA in response to MBP 84-102 peptide or to a combination of PMA + ionomycin or peptide + PMA. In contrast, the anergized T cell clone failed to synthesize IL-2, IL-4, and IFN-γ mRNA in response to MBP 84-102 peptide alone or to the peptide in combination with PMA, while synthesizing significant cytokine mRNA in response to PMA + ionomycin

stimulation (Fig. 7 A). The results of the Northern analysis of IL-2 mRNA were confirmed by an IL-2 bioassay using HT-2 cells (Fig. 7 B). Anergized T cells activated with peptide antigen in a secondary stimulation did not secrete IL-2, whereas the nonanergized did secrete IL-2, as measured by the proliferation of HT-2 cells.

Secondary stimulation

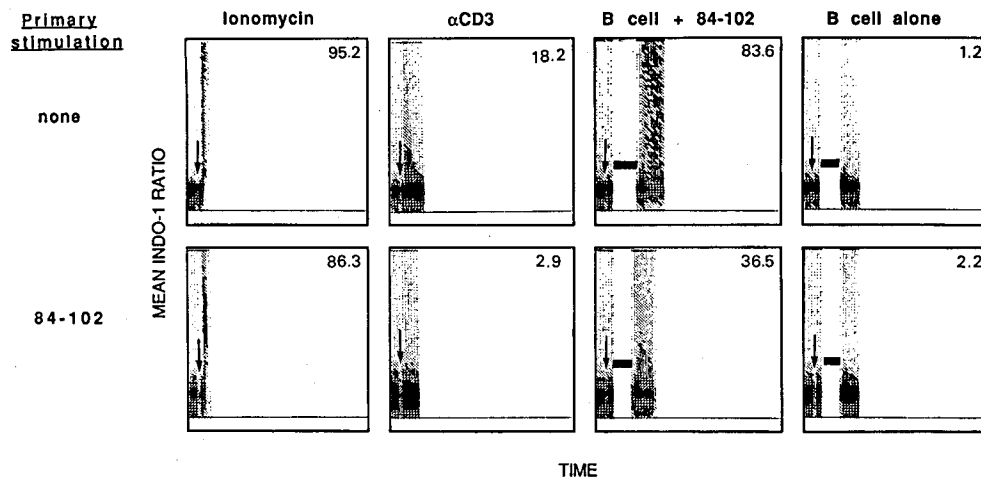


Figure 6. Anergized T cells are inhibited in their ability to release $[Ca^{2+}]_i$ to antigenic stimulation. Ob.1A12.8 was cultured $\pm 5 \mu\text{g/ml}$ 84-102 for 7 d, washed, and loaded with $2 \mu\text{g/ml}$ Indo-1 for analysis of $[Ca^{2+}]_i$. Secondary stimulation by ionomycin ($100 \mu\text{g/ml}$), αCD3 ($1/30$ ascites dilution), or B cells (9010) pulsed + $100 \mu\text{g/ml}$ 84-102 was added to flow cytometer at point designated by arrows. For APC stimulation, cells were removed from flow cytometer and centrifuged for 1 min (represented by filled bars). Percent response for each sample represents percent of cells above baseline (poststimulus minus prestimulus).

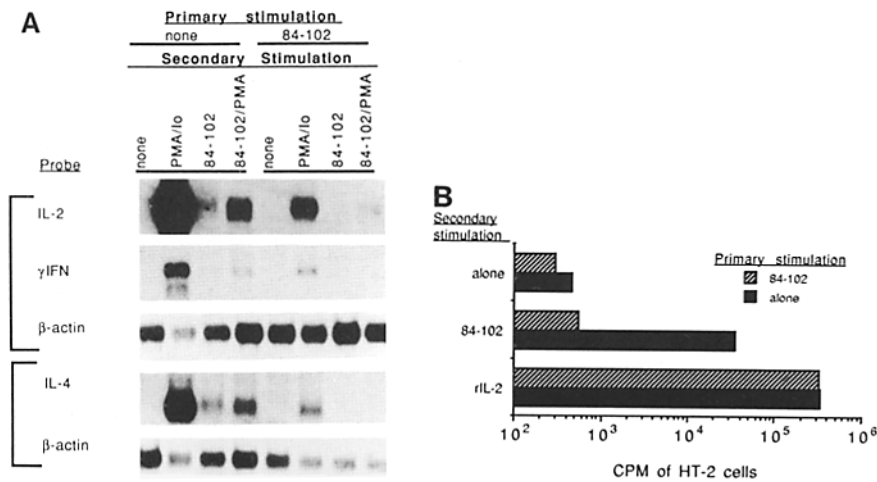


Figure 7. Cytokine production by energized T cells. (A) T cell clone Ob.1A12.8 was cultured with or without 84-102 for 2 d, washed, and stimulated with or without 10 ng/ml PMA + 1 μg/ml ionomycin or 5 μg/ml 84-102 ± 10 ng/ml PMA for 4 h at 37°C. Total cellular RNA was extracted by the RNazol B method for Northern analysis. Brackets signify two different Northern blots of identical samples each hybridized with a β-actin control probe in addition to cytokine-specific probes. Size of mRNA was determined by standards to be 0.9 kb IL-2, 1.3 kb IFN-α, 0.7 kb IL-4, and 1.8 kb β-actin. (B) Ob.1A12.8 was cultured for 2 d ± 84-102, washed, and stimulated with 84-102 or rIL-2 as a positive control. Supernatants were collected after 20 h of secondary culture and diluted 1:3 in cultures of HT-2 cells. Proliferation of HT-2 cells represents the presence of IL-2 in supernatant of 84-102-stimulated nonenergized T cells and was maximal with the rIL-2-positive control.

Anergy Is Induced Only in Peptide-specific T Cell Clones. As the induction of anergy seemed to involve a dominant negative signal that was not overcome by the addition of accessory cells, it was important to exclude the possibility that peptide binding to MHC class II may deliver a negative signal to T cells. To assess the involvement of the TCR in the induction of anergy, we compared the ability of an MBP-reactive T cell vs. a T cell clone, derived from the same individual by direct single cell cloning, to become energized by MBP peptide 84-102. As shown in Table 2, MBP-reactive T cell clone 2Hy.1G11.7 stimulated by peptide 84-102 was rendered unresponsive to secondary stimulation by both 84-102 and αCD3 + PMA, but not IL-2. In contrast, the MHC-matched clone Hy.6 did not respond to 84-102 regardless of the primary stimulation, and the response to αCD3 + PMA was unchanged by pretreatment with peptide.

Discussion

Traditional APCs such as macrophages, B cells, and dendritic cells constitutively express MHC class II and are able to process and present protein antigen to CD4⁺ T cells (28).

Human T cells that express MHC class II after activation are able to present peptide or degraded antigen but not normally process whole antigen, suggesting that they act as “non-traditional” APCs (22, 29–31). Although T cell presentation of MBP 84-102 results in a proliferative response in a primary proliferation assay, here we show that T cells stimulated with peptide antigen are unresponsive to secondary stimulation by either antigen or TCR/CD3 crosslinking, but not IL-2. Moreover, long-lasting antigen unresponsiveness was induced with unbound peptide but not peptide-pulsed B cells or DR2⁺ L cell transfectants, suggesting that the unresponsiveness was not merely due to an unresponsive refractory period after prior stimulation, as has been suggested as a mechanism of high-dose tolerance (9). We refer to this unresponsiveness as anergy because the T cells are unresponsive to antigenic stimulation while remaining IL-2 responsive, which were the original criterion established to define the term (8).

T cell tolerance can be induced in vitro by chemically fixed APC or immobilized αCD3 mAb, where a signal is delivered through the TCR/CD3 complex without a second essential costimulator signal (6–8). It has been shown that the CD28 molecule on T cells, interacting with B7 on B cells

Table 2. Non-MBP-reactive T Cell Clone from the Same Subject Does Not Become Anergized to 84-102

Secondary stimulation	Primary stimulation			
	2Hy.1G11.7		Hy.6	
	Alone	84-102	Alone	84-102
Alone	168 ± 37	162 ± 35	95 ± 18	102 ± 21
84-102	214,059 ± 84,810	1,103 ± 797	69 ± 25	74 ± 16
IL-2	183,662 ± 1,336	117,032 ± 1,501	361,087 ± 13,097	565,394 ± 10,622
Anti-CD3 + PMA	216,481 ± 18,781	4,244 ± 646	514,303 ± 36,834	444,584 ± 19,609

MBP-reactive T cell clone 2Hy.1G11.7 and PHA-derived non-MBP-reactive T cell clone Hy.6 were cultured for 2 d in the presence or absence of 2 μM 84-102. After primary culture, T cells were washed and assayed for proliferation to 84-102 (2 μM), rIL-2 (10³ u/ml), or anti-CD3 mAb (OKT3) (1/100 ascites dilution) + PMA (10 ng/ml). Anergic responses are highlighted in bold.

and activated macrophages, is a component of the costimulatory pathway necessary for T cell activation and postulated that the absence of B7 costimulation may lead to anergy (14–16). However, T cell anergy induced by self T cell presentation of MBP peptide appears to occur in the presence of costimulation because the addition of either B7 transfectants or EBV-transformed B cells, which express high levels of B7, were unable to prevent the induction of unresponsiveness. Moreover, B cells that have the appropriate MHC and act as nontolerogenic APCs in the absence of free antigen, and presumably compete for presentation of peptide antigen with T cells during culture, are not able to overcome the induction of unresponsiveness. These results suggest that the induction of anergy by T cell presentation of peptide antigen results in a negative signal rather than the lack of a positive costimulation. These results have practical implications for the growth of antigen-specific T cell lines and clones that should be restimulated in the absence of free peptide antigen to prevent the loss of antigen responsiveness. This dominant negative signal does not appear to be due to a signal through MHC class II because non-MBP-reactive T cell clones bearing MHC class II do not become anergized by MBP peptide (Table 2). Further, incubation of MBP-reactive T cell clone 2Hy.1G11.7 with an anti-DR mAb either alone or with secondary crosslinking with GAMIG did not anergize the T cell clone (data not shown). In total, these results demonstrate that anergy cannot be explained by a direct negative signal through MHC class II.

The mechanism of anergy induced by T cell presentation of autoantigen was examined. Anergized T cells have a marked diminution in their ability to release $[Ca^{2+}]_i$ in response to either APCs or α CD3 mAb crosslinking. In addition, treatment with the combination of PMA and ionomycin completely restored the proliferation and partially restored the cytokine production of anergized T cells, suggesting that the signaling events after protein kinase C (PKC) activation and $[Ca^{2+}]_i$ release are not defective. The slight $[Ca^{2+}]_i$ increase in anergized T cells stimulated with APCs (Fig. 6) suggests that there may be a signal in anergized T cells, exceeding proximal signaling events, that is perhaps involved in the maintenance of anergy. However, this level of $[Ca^{2+}]_i$ is apparently not enough to reach the threshold needed for proliferation.

Thus, the state of anergy as defined by alterations in signal transduction in our system are different from studies of *in vitro* clonal anergy by Mueller et al. (32, 33) where no signaling defects were observed in membrane proximal events. The mechanism of T cell anergy induced by T cell presentation of antigen appears similar to studies in the murine system of transient activation-induced anergy, which can be reversed by treatment with calcium ionophore with antigen or PMA (34). Similarities also exist with studies of transgenic mice in which autoreactive T cells that escape into the periphery fail to release $[Ca^{2+}]_i$ in response to TCR signaling but are able to proliferate in response to the combination of PMA and ionomycin (21). Therefore, there appear to be at least two different states of functionally defined anergy. Just as

cell transformation can occur by the alteration of one of many different molecules that regulate a cell's signal transduction pathway, the phenomenon of T cell anergy may be achieved by several different mechanisms that affect different stages of T cell activation.

O'Hehir et al. (35, 36) have shown that T cell unresponsiveness induced by peptide or superantigen can be characterized by a decrease in the surface expression of CD3 together with an increase in CD25 16 h after the addition of peptide antigen. We also observe a moderate decrease in TCR/CD3 expression and increase in CD25 expression at this early time point, presumably due to T cell activation (data not shown). However, we find that by 4 d, anergized T cells have equivalent cell surface density of CD3 as nonanergized T cells (Fig. 4). This indicates that anergy cannot be explained simply as the reduction of cell surface TCR/CD3.

As we have seen with the majority of human T cell clones derived from peripheral blood (24), T cell clone Ob.1A12.8 appears to be a Th0 phenotype in its ability to produce cytokines of both the Th1 and Th2 subsets in response to mitogen stimulation (37). Anergized T cells were unable to synthesize IL-2, IL-4, or IFN- γ mRNA, or secrete measurable IL-2 in response to antigenic stimulation either with or without PMA, while the addition of both ionomycin and PMA generated cytokine synthesis. These results are consistent with both proliferation and Ca^{2+} flux data, suggesting that the block in T cell signaling is in an event preceding Ca^{2+} mobilization. The negative signal generated by T cells in the response to peptide antigen is yet to be defined, as is the actual biochemical signaling event that is altered in these anergized T cells.

A major question in immunology relates to how activated autoantigen-reactive T cells are regulated during an inflammatory response. In previous work, we have shown that T cells are able to present peptide antigen and partially degraded native MBP, although they are unable to process and present highly purified MBP (22). This led us to speculate that activated T cells in an inflammatory site, such as a demyelinating plaque in the brain, may be able to present fragments of MBP to other T cells at the site. The demonstration that T cell presentation of peptide antigen leads to clonal unresponsiveness suggests that this mechanism of tolerance may have evolved to prevent autoreactive T cells in an inflammatory site from clonally expanding to damaged self-tissue. A high extracellular concentration of degraded protein would be characteristic of self-antigen, while foreign antigen after the initial *in situ* immune response would be present at a lower concentration and become internalized into traditional APCs for processing and presentation. In this way, inappropriate antigen presentation by T cells that can present but not process the antigen would lead to anergy instead of activation of the responding T cell clone in an inflammatory site where degraded self-antigen is available.

In conclusion, we present evidence for a mechanism to explain the initial observation of Lamb et al. (10, 11) that pretreatment of T cell clones with peptide antigen leads to antigen unresponsiveness. Anergy is related to the expression of MHC

class II by activated human T cells, which are able to present peptide antigen to autologous T cells. Although this interaction leads to a primary stimulation of the T cells, they are rendered unresponsive to subsequent stimulation within 24 h of peptide pretreatment. The signaling defect in anergized

T cells is membrane proximal because peptide-treated T cell clones are inhibited in their ability to release $[Ca^{2+}]_i$, produce cytokines, and proliferate to antigenic stimuli, but exhibit a normal proliferative response to treatment with PMA and ionomycin.

We thank D. Benjamin and B. Lacet for technical assistance, P. Lopez for flow cytometry, and A. Abbas for helpful discussions.

This work was supported by National Institutes of Health grant RO1 NS-24247, and a grant from the National Multiple Sclerosis Society.

Address correspondence to Janine M. LaSalle, Center for Neurologic Diseases, Brigham and Women's Hospital, 221 Longwood Avenue, Boston, MA 02115.

Received for publication 28 January 1992 and in revised form 6 April 1992.

References

1. Kappler, J.W., U.D. Staerz, J. White, and P.C. Marrack. 1988. Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. *Nature (Lond.)* 332:35.
2. MacDonald, H.R., R. Schneider, R.K. Lees, R.C. Howe, H. Acha-Orbea, H. Festenstein, R.M. Zinkernagel, and H. Hengartner. 1988. T cell receptor V β use predicts reactivity and tolerance to Mlsa-encoded antigens. *Nature (Lond.)* 332:40.
3. Kappler, J.W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell* 49:273.
4. Kieselow, P., H. Bluthman, U.D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T cell receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. *Nature (Lond.)* 333:742.
5. Ota, K., M. Matsui, E.L. Milford, G.A. Mackin, H.L. Weiner, and D.A. Hafler. 1990. T cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature (Lond.)* 346:183.
6. Jenkins, M.K., J.D. Ashwell, and R.H. Schwartz. 1988. Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen presenting cells. *J. Immunol.* 140:3324.
7. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. Clonal expansion versus clonal inactivation: A costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu. Rev. Immunol.* 7:445.
8. Jenkins, M.K., D.M. Pardoll, J. Mizuguchi, H. Quill, and R.H. Schwartz. 1987. T-cell unresponsiveness in vivo and in vitro: fine specificity of induction and molecular characterization of the unresponsive state. *Immunol. Rev.* 95:113.
9. Suzuki, G., Y. Kawase, S. Koyasu, I. Yahara, Y. Kobayashi, and R.H. Schwartz. 1988. Antigen-induced suppression of the proliferative response of T cell clones. *J. Immunol.* 140:1359.
10. Lamb, J.R., B.J. Skidmore, N. Green, J.M. Chiller, and M. Feldman. 1983. Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. *J. Exp. Med.* 157:1434.
11. Lamb, J.R., and M. Feldmann. 1984. Essential requirement for major histocompatibility complex recognition in T-cell tolerance induction. *Nature (Lond.)* 308:72.
12. Kennedy, M.K., M.C. Dal Canto, J.L. Trotter, and S.J. Miller. 1988. *J. Immunol.* 141:2986.
13. Williams, I.F., and E.R. Unanue. 1990. Costimulatory requirements of murine Th1 clones. The role of accessory cell-derived signals in response to anti-CD3 antibody. *J. Immunol.* 145:85.
14. Gimmi, C.D., G.J. Freeman, J.G. Gribben, K. Sujgita, A.S. Freedman, C. Morimoto, and L.M. Nadler. 1991. B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. *Proc. Natl. Acad. Sci. USA.* 88:6575.
15. Linsley, P.S., W. Brady, L. Grosmaire, A. Aruffa, N.K. Darnell, and J.A. Ledbetter. 1991. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J. Exp. Med.* 173:721.
16. Freedman, A.S., G. Freeman, J.C. Horowitz, J. Daley, and L.M. Nadler. 1987. B7, a B cell-restricted antigen that identified preactivated B cells. *J. Immunol.* 139:3260.
17. Burkly, L.C., D. Lo, O. Kanagawa, R.L. Brinster, and R.A. Flavell. 1989. T-cell tolerance by clonal anergy in transgenic mice with nonlymphoid expression of MHC class II I-E. *Nature (Lond.)* 342:564.
18. Lo, D., L.C. Burkly, G. Widera, C. Cowing, R.A. Flavell, R.D. Palmiter, and R.L. Brinster. 1988. Diabetes and tolerance in transgenic mice expressing class II MHC molecules in pancreatic beta cells. *Cell.* 53:159.
19. Webb, S., C. Morris, and J. Sprent. 1990. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell.* 63:1249.
20. Ramsdell, F., T. Lantz, and B.J. Fowlkes. 1989. A nondeletional mechanism of thymic self tolerance. *Science (Wash. DC)* 246:1038.
21. Blackman, M.A., T.H. Finkel, J. Kappler, J. Cambier, and P. Marrack. 1991. Altered antigen receptor signalling in anergic T cells from self-tolerant T-cell receptor β -chain transgenic mice. *Proc. Natl. Acad. Sci. USA.* 88:6682.
22. LaSalle, J.M., K. Ota, and D.A. Hafler. 1991. Presentation of

- autoantigen by human T cells. *J. Immunol.* 147:774.
23. Grosveld, F.G., T. Lund, E.J. Murray, A.L. Mellor, H.H.M. Dahl, and R.A. Flavell. The construction of cosmid libraries which can be used to transform eukaryotic cells. *Nucleic Acids Res.* 10:6715.
 24. Brod, S.A., D. Benjamin, and D.A. Hafler. 1991. Restricted T cell expression of IL-2/IFN- γ mRNA in human inflammatory disease. *J. Immunol.* 147:810.
 25. Freeman, G.J., G.S. Gray, C.D. Gimmi, D.B. Lombard, L.J. Zhou, M. White, J.D. Fingerth, J.G. Gribben, and L.M. Nadler. 1991. Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. *J. Exp. Med.* 174:625.
 26. Altman, A., K.M. Coggeshall, and T. Mustelin. 1990. Molecular events mediating T cell activation. *Adv. Immunol.* 48:227.
 27. Bierer, B.E., P.S. Mattila, R.F. Standaert, L.A. Herzenberg, S.J. Burakoff, G. Crabtree, and S.L. Schreiber. 1990. Two distinct signal transmission pathways are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. *Proc. Natl. Acad. Sci. USA.* 87:9231.
 28. Unanue, E.R., and P.M. Allen. 1987. The basis for the immunoregulatory role of macrophages and other accessory cells. *Science (Wash. DC).* 236:551.
 29. Hewitt, C.R.A., and M. Feldman. 1989. Human T cell clones present antigen. *J. Immunol.* 142:1429.
 30. Lanzavecchia, A., E. Roosnek, T. Gregory, P. Berman, and S. Abrignani. 1988. T cells can present antigens such as HIV gp120 targeted to their own surface molecules. *Nature (Lond.)* 334:530.
 31. Gerrard, T.L., D.J. Volkman, C.H. Jurgensen, and A.S. Fauci. 1986. Activated human T cells can present denatured antigen. *Hum. Immunol.* 17:416.
 32. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. *J. Immunol.* 142:2617.
 33. Mueller, D.M., M.K. Jenkins, L. Chiodetti, and R.H. Schwartz. 1990. An intracellular calcium increase and protein kinase C activation fail to initiate T cell proliferation in the absence of a costimulatory signal. *J. Immunol.* 144:3701.
 34. Otten, G., K.C. Herold, and F.W. Fitch. 1987. Interleukin 2 inhibits antigen-stimulated lymphokine synthesis in helper T cells by inhibiting calcium-dependent signalling. *J. Immunol.* 139:1348.
 35. O'Hehir, R.E., H. Yssel, S. Verma, J.E. de Vries, H. Spits, and J.R. Lamb. 1991. Clonal analysis of different lymphokine production in peptide and superantigen induced T cell anergy. *Int. Immunol.* 3:819.
 36. O'Hehir, R.E., and J.R. Lamb. 1990. Induction of specific clonal anergy in human T lymphocytes by *Staphylococcus aureus* enterotoxins. *Proc. Nat. Acad. Sci. USA.* 87:8884.
 37. Street, N., J.H. Schumacher, T.A.T. Fong, H. Bass, D.F. Fiorentino, J.A. Leverach, and T.R. Mossman. 1990. Heterogeneity of mouse helper T cells: evidence from bulk cultures and limiting dilution cloning for precursors of Th1 and Th2 cells. *J. Immunol.* 144:1629.