Th2 Cell Clonal Anergy as a Consequence of Partial Activation

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Summary

We have demonstrated Th2 clonal anergy as a consequence of partial T cell activation by immunogenic peptide and chemically fixed APC, as well as by altered peptide ligand and live antigen-presenting cells (APC). Either stimulation resulted in a profound inability of the T cells to proliferate upon restimulation with antigen and functional APC, a similar phenomenon to that found with Th1 cells. The anergic state was long lasting and was restricted to proliferation, since the T cells retained the ability to produce cytokines upon restimulation, albeit at slightly reduced levels. Th2 anergy induction was inhibited by cyclosporine A, but not by provision of exogenous costimulation or growth factors. The data presented unify Th1 and Th2 cells with regard to anergy and suggest that the fundamental control during anergy for both subsets is prevention of clonal expansion, thus blocking amplification of the immune response.

$CD4^+$ T cells require TCR engagement by an immunogenic peptide bound to a class II MHC molecule, as well as a costimulus provided by the APC, for successful activation leading to clonal expansion (1, 2). Although the nature of the costimulus has not been completely elucidated, substantial evidence has accumulated implicating the B7-CD28 interaction in the Th1 subset (3), whereas IL-1 has been shown to have costimulatory activity for Th2 cells (4–7). In the Th1 subset, it has been clearly shown that TCR engagement without costimulation does not provide adequate signaling to induce proliferation (8–10). Interestingly, such an interaction leads to a state of profound unresponsiveness in these T cells upon restimulation, a phenotype known as T cell anergy (9–12). This unresponsive state appears to result from TCR triggering without T cell proliferation (13) and can be prevented by providing exogenous costimulation during the initial TCR engagement (3, 14). More recently, we have demonstrated that stimulation of Th1 clones with altered peptide ligands (APL)[1] and live APC, a combination that partially activates the T cells but does not induce proliferation, also results in unresponsiveness to future stimulation with the immunogenic peptide (15). Addition of exogenous costimulation does not prevent anergy from occurring in this situation, suggesting that a signaling event more proximal to the TCR is responsible for inducing unresponsiveness here (16). Although the intracellular events involved are not yet revealed, it is clear that T cells of the Th1 subset can be made functionally unresponsive for prolonged periods of time.

In contrast to the extensive studies of Th1 cell anergy, few investigations have been published that use similar protocols in the Th2 subset. From the limited data available, it has been generally accepted that Th2 cells are not susceptible to anergy induction after TCR occupancy without costimulation (2, 17, 18). Moreover, anti-CD3 antibody induces unresponsiveness to IL-2–driven proliferation in Th1 but not Th2 clones (9), and anergy-inducing stimuli do not result in an autocrine growth factor production defect in Th2 cells (19). Although the reason for this contrast in phenotype is unclear, one could ascribe it to the different intracellular signaling pathways activated after stimulation of the two T cell subsets, use of different costimulators or growth factors, or a combination of these (5, 6, 20). One study has demonstrated a correlation between the presence of IL-4 and the resistance to tolerance induction, both at the clonal level and in naive T cell populations (18).

We reasoned that if anergy plays a significant role in T cell tolerance, then anergy should also be inducible in Th2 cells. We decided to explore the susceptibility to tolerance induction of Th1 and Th2 clones with identical antigen/MHC specificity, namely, Hb(64-76)/I-E^k. We have previously shown that the Th1 clones could be made anergic after presentation of immunogenic peptide and chemically fixed APC or by presentation of APL and live APC (15). In this report we show the results obtained when analogous experiments were undertaken with the Th2 cells. Our data demonstrate that antigen presentation by chemically fixed APC does indeed result in subsequent anergy of these Th2 cells to restimulation with functional APC and antigen. Moreover, we

[1] *Abbreviations used in this paper:* APL, altered peptide ligands; ECD1, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HEL, hen egg white lysozyme; RT, reverse transcriptase.

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show that stimulation of these T cells with live APC and APL, which stimulates cytokine production but not proliferation, also results in an anergic phenotype upon restimulation with the immunogenic peptide. This extends our earlier observations with Th1 clones to include Th2 clones and implies that the anergic state is important for both Th1 and Th2 cells.

Materials and Methods

Mice. Female CBA/J and B10.BR/SgSnJ (referred to as B10.BR) mice were purchased from National Cancer Institute (Bethesda, MD) or The Jackson Laboratory (Bar Harbor, ME) and used at 5–10 wk of age.

Ag and Antisera. The Hb^d(64-76) peptide, APL, and conalbumin peptide were synthesized with a RaMPS apparatus (DuPont, Wilmington, DE), purified by reverse-phase HPLC, and amino acid content confirmed by analysis on an amino acid analyzer (model 6300, Beckman, Palo Alto, CA). APL is the name ascribed to analogs of immunogenic peptides in which the TCR contact residues have been manipulated. These peptides do not stimulate T cell–proliferative responses, but they do bind to the MHC molecules with similar affinities as the wild-type peptide, stimulating some, but not all, TCR-mediated effector functions (16). The amino acid sequence for the Hb(64-76) peptide shown in the one-letter amino acid code is GKKVITAFNEGLK. The APL each contain a single amino acid substitution; for example, the Asp73 APL contains an aspartate in place of glutamate at position 73. The amino acid sequence for the conalbumin peptide shown in the one-letter amino acid code is LLHWGAIEWEGIESG (21). mAb specific for IL-2 (S4B6; 22), IL-4 (11B11; American Type Culture Collection, Rockville, MD) and CD3ϵ (23) were used as culture supernatants from the respective hybridoma cell lines.

Cell Lines. We have previously described the generation and characterization of a panel of Hb-reactive Th clones, including the Th2 2.102 and EW5X clones (24). The Th clones were propagated on a 2-wk restimulation protocol by use of irradiated (2,000 rad) CBA/J splenocytes as a source of APC and endogenous antigen in RPMI 1640 medium containing 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM glutamine, 50 μg/ml gentamycin, Hepes buffer, 2-ME (2×10^{-5} M), and IL-2 (20 U/ml, supernatants from P815 transfectants expressing the gene for murine IL-2; 25). The T cell hybridoma, G2, was made by use of a standard fusion protocol with the Th2 clone, 2.102, (7 d after activation) and the $\alpha^-\beta^-$ BW5147 fusion partner (26, 27). The Th2 clone D10 has been described elsewhere (28). It was propagated as described above, with conalbumin protein (100 μg/ml) as antigen. The CH27 B cell lymphoma, which expresses $H\text{-}2^k$, B7, and ICAM-1, was used as an APC source for the tolerance induction assays using live APC (27).

Preparation of Fixed APC. B10.BR spleen cells were fixed by two different methods. The chemical cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECDI; Calbiochem-Behring Corp., La Jolla, CA) was used in the experiment shown in Fig. 2 *A* only; paraformaldehyde was used in all other experiments. For ECDI fixation, 10^8 spleen cells were incubated for 1 h on ice in 0.44 ml 0.9% NaCl containing 75 mM ECDI, and then washed extensively. For paraformaldehyde fixation, spleen cells (5×10^7 cells/ml) were incubated in paraformaldehyde (1% in HBSS) for 10 min at room temperature, washed three times, incubated for 30 min at 37°C in a 0.1-M lysine solution, and finally washed three more times in HBSS. No differences in the efficacy of these two fixation protocols were observed.

Proliferation Assays. The proliferative responses of the Th2 clones were performed in 96-well flat-bottomed plates in 200 μl RPMI 1640 medium containing the clones (5×10^4 cells/well), irradiated or chemically fixed B10.BR spleen cells (2,000 rad, 5×10^5 cells/well), and Hb(64-76) peptide (0–10 μM) or conalbumin peptide (0–316 μM). Wells were pulsed with [^{3}H]TdR (0.4 μCi/well) after 72 h of culture and harvested 18–24 h later. In some experiments, anti–IL-2 (50 μl/well) or anti–IL-4 (25 μl/well) mAb culture supernatants or murine rIL-1β (50 pg/ml, a generous gift of David D. Chaplin, Washington University, St. Louis, MO) were added for the duration of the assay.

Tolerance Induction Assays. Th2 cells ($2.5-10 \times 10^5$ cells/well) and fixed or irradiated B10.BR spleen cells (5×10^6 cells/well) were incubated at 37°C alone or with 10 μM Hb(64-76) peptide or 316 μM conalbumin peptide, either overnight (Fig. 2, *A* and *C*) or for the times indicated, in 24-well tissue culture plates in a final volume of 1 ml. T cells were then separated from APC as described (15), before being challenged in a proliferation assay using either 5×10^4 T cells/well and Hb(64-76) as antigen or anti-CD3ϵ mAb (2C11) culture supernatant and IL-2 (20 U/ml). Where indicated, cyclosporine A (1 μg/ml; Sandoz, Basel, Switzerland), rIL-1β (50 pg/ml), IL-2, or IL-4 (20 units/ml) (25) were added during the tolerance induction stage. For tolerance induction with live APC and analog peptides, the protocol was as above, except that mitomycin C–treated (77 μl/ml, 90 min at 37°C) CH27 B lymphoma cells were used as APC (3×10^5/well), and analog peptides were added as indicated (100 μM).

Lymphokine Assays. The lymphokine responses of the Th2 clones were assessed by use of 24-h supernatants from cultures of proliferation assays. IL-4 was quantitated in a bioassay as proliferation of the IL-2/IL-4–dependent CTLL-2 cell line (29) or the IL-4–dependent 6I4 cell line (a kind gift of Osami Kanagawa, Washington University). Briefly, the cytokine-dependent cells (5×10^3/well) were incubated with test supernatants (25 μl) for 48 h. [^{3}H]TdR was included during the final 20 h. Where indicated, anti–IL-2 (50 μl) or anti–IL-4 (25 μl) culture supernatants were also added for the duration of the bioassay. IL-3 was quantitated as proliferation of the IL-3–dependent GG-1.12 cell line (30) and assayed as above, with 10^4 GG-1.12 cells and 50-μl test supernatants.

mRNA Detection. Th clones and hybridomas (10^6 cells/well) were incubated in 24-well tissue culture plates at 37°C alone, with immobilized anti-CD3ϵ mAb (200 μl/well prebound for 6 h, then the excess washed off with HBSS), or with CH27 B lymphoma cells (3×10^5/well) and 10 μM Hb(64-76) for 14 h, after which total RNA was extracted as described (31), and dissolved in water. Cytokine mRNA was detected by reverse transcriptase (RT) of the mRNA, followed by a PCR. First, strand cDNA was made in a 20-μl reaction, by use of 1 μg of total RNA as template, an oligo-dT primer, and the superscript RT enzyme (GIBCO BRL, Gaithersburg, MD). The PCR (32) was then used to amplify cDNA of IL-2, IL-4, and the Vβ1 TCR chain by use of a thermocycler (*Temp. Tronic*; Thermolyne, Dubuque, IA). All oligonucleotide primers used to amplify cytokine cDNA span introns and the sequences and amplification conditions are provided elsewhere (33). Nucleotide sequences for primers used to amplify Vβ1 cDNA are as follows: Vβ1 primer sequence is 5'GTCCTGAATTCAGCTGCAGGCTTC-TCCT 3'; Cβ1 primer sequence is 5'GAGGGTAGCCTTTTG-TTTGTTTGCAAT 3' (a gift from Kenneth M. Murphy, Washington University). All oligonucleotide primers were gener-

ated with a DNA synthesizer (Oligo 1000; Beckman, Fullerton, CA). Amplified bands were analyzed on 3% agarose gels (NuSieve; FMC Corp., Rockland, ME).

Results

Antigen Presentation by Chemically Fixed Splenocytes Does Not Activate Th2 Cell Proliferation. Although presentation to Th1 cells of an antigenic peptide by chemically fixed spleen cell APC does not support clonal expansion, reports suggest that Th2 cells can proliferate well to such a stimulus (17). We decided to directly examine the Th2 response to antigen and fixed APC, since the published data suggested a striking difference in activation requirements by the two Th cell subsets. The Th2 clone 2.102 recognizes the immunogenic peptide of the murine hemoglobin β^d minor chain, Hb(64-76), bound to an I-E^k molecule, and has been exclusively characterized (24, 34). Irradiated B10.BR splenocytes and Hb(64-76) peptide stimulated a vigorous proliferative response from 2.102 cells. In marked contrast, however, presentation of the immunogenic peptide by chemically fixed spleen cells did not induce any detectable proliferation from these T cells (Fig. 1 *A*). This phenotype was surprising since it differed from that reported for other Th2 clones (17). However, this lack of proliferation was not simply due to a shift in the antigen dose curve, since presentation of up to 100 μM peptide by these APC (>100-fold excess of the 50% maximal response using irradiated spleen cell APC) did not stimulate any clonal expansion of the 2.102 cells (data not shown). Whereas the signal delivered to the T cells by antigen and fixed APC was inadequate to stimulate proliferation, the amount of IL-4 produced was normal (or only slightly decreased in some experiments), whereas IL-3 production was somewhat reduced (Fig. 1, *B* and *C*). This phenotype resembles that of Th1 cell reactivity to antigen presentation by fixed APC, and therefore suggests the absence of a costimulus that is required for proliferation and maximal IL-3 production, but not IL-4 production.

Antigen Presentation by Chemically Fixed Splenocytes Induces Anergy of Th2 Cells to Subsequent Stimulation with Functional APC. The observation that chemically fixed APC failed to stimulate Th2 cell proliferation, but supported cytokine production, raised the possibility that antigen recognition in this way might lead to unresponsiveness in these T cells. To test this, 2.102 cells were preincubated overnight with chemically fixed B10.BR splenocytes, with or without antigen, and restimulated with fresh irradiated spleen cells and antigen. Whereas T cells that had been preincubated with fixed APC alone were able to proliferate normally during the secondary stimulation, those that had been preincubated with fixed APC and antigen were almost completely unresponsive to restimulation with fresh spleen cells and antigen (Fig. 2 *A*). This inability to proliferate upon restimulation was a long-lasting phenotype, since the T cells remained unrespon-

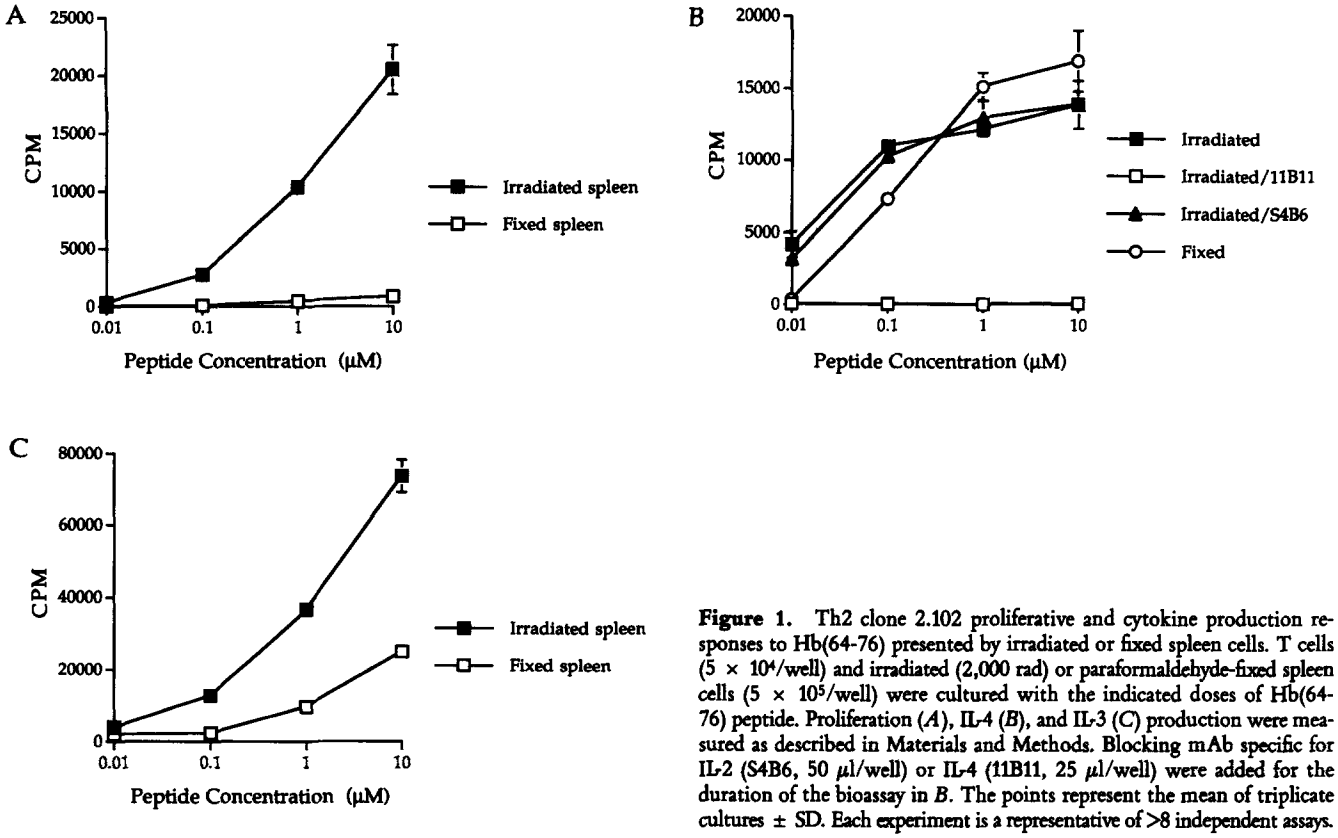


Figure 1. Th2 clone 2.102 proliferative and cytokine production responses to Hb(64-76) presented by irradiated or fixed spleen cells. T cells (5×10^4/well) and irradiated (2,000 rad) or paraformaldehyde-fixed spleen cells (5×10^5/well) were cultured with the indicated doses of Hb(64-76) peptide. Proliferation (*A*), IL-4 (*B*), and IL-3 (*C*) production were measured as described in Materials and Methods. Blocking mAb specific for IL-2 (S4B6, 50 μl/well) or IL-4 (11B11, 25 μl/well) were added for the duration of the bioassay in *B*. The points represent the mean of triplicate cultures ± SD. Each experiment is a representative of >8 independent assays.

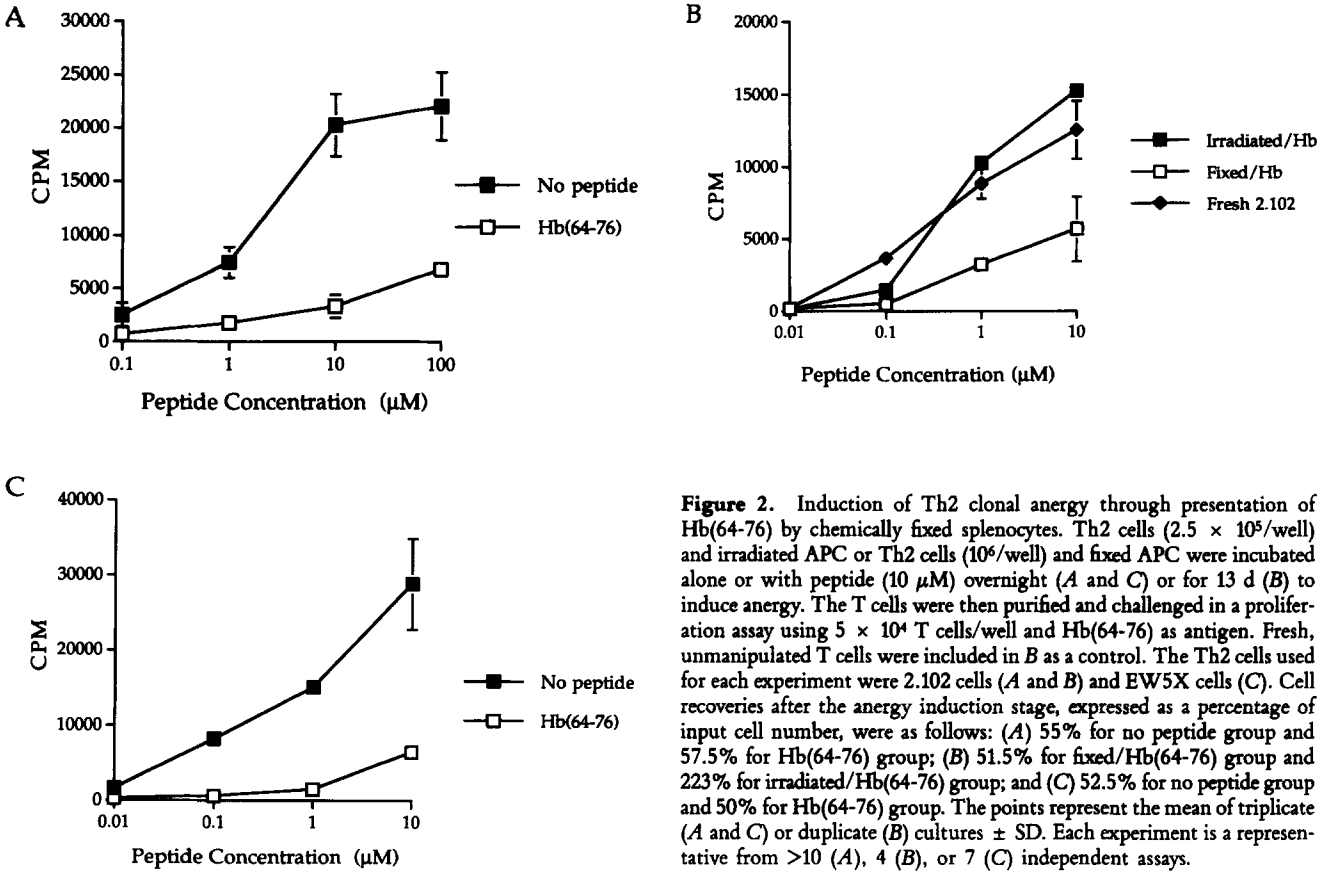


Figure 2. Induction of Th2 clonal anergy through presentation of Hb(64-76) by chemically fixed splenocytes. Th2 cells (2.5×10^5/well) and irradiated APC or Th2 cells (10^6/well) and fixed APC were incubated alone or with peptide (10 μM) overnight (*A* and *C*) or for 13 d (*B*) to induce anergy. The T cells were then purified and challenged in a proliferation assay using 5×10^4 T cells/well and Hb(64-76) as antigen. Fresh, unmanipulated T cells were included in *B* as a control. The Th2 cells used for each experiment were 2.102 cells (*A* and *B*) and EW5X cells (*C*). Cell recoveries after the anergy induction stage, expressed as a percentage of input cell number, were as follows: (*A*) 55% for no peptide group and 57.5% for Hb(64-76) group; (*B*) 51.5% for fixed/Hb(64-76) group and 223% for irradiated/Hb(64-76) group; and (*C*) 52.5% for no peptide group and 50% for Hb(64-76) group. The points represent the mean of triplicate (*A* and *C*) or duplicate (*B*) cultures ± SD. Each experiment is a representative from >10 (*A*), 4 (*B*), or 7 (*C*) independent assays.

sive 13 d after the initial stimulation with fixed APC and antigen (Fig. 2 *B*). In contrast, T cells that had been preincubated with irradiated spleen cells and antigen proliferated as well as freshly isolated 2.102 cells at this timepoint (Fig. 2 *B*). The observed lack of proliferation upon antigenic challenge could not be explained by cell death, since equivalent numbers of cells were recovered whether or not peptide was present during the initial stimulus with fixed APC (Fig. 2 *legend*). Moreover, both anergic and nonanergic Th2 cells could proliferate equivalently in response to exogenously added IL-2 when combined with anti-CD3 mAb stimulation to upregulate surface levels of the IL-2R, further indicating that cell death did not account for the unresponsiveness to antigen stimulation in the anergic T cells (Table 1).

Th2 Cells Are Made Anergic after Stimulation by Altered Peptide Ligand and Live APC. We have previously shown that stimulation of Th1 clones with live APC and APL, which allows partial T cell activation but not proliferation, leads

Table 1. *Comparison of Proliferation of Anergic versus Nonanergic T Cells to Antigen or Exogenous IL-2*

Preincubation condition (with fixed APC)	Restimulation (cpm)			
	Hb(64-76)/APC		anti-CD3ε + IL-2 (20 U/ml)	
	1 μM	0.1 μM	5 μl	2 μl
No peptide	12,649	10,732	43,240	43,100
Hb(64-76)	5,853	4,563	46,004	43,829

2.102 cells (10^6/well) and fixed spleen cells were incubated overnight alone or with indicated peptide (10 μM). T cells were then recovered and assayed for their ability to proliferate in response to peptide or exogenous IL-2. Anti-CD3 mAb was included with the IL-2 stimulus to upregulate cell surface levels of IL-2R, since the T cells from both groups responded poorly to IL-2 alone. Cell recoveries after anergy induction, expressed as a percentage of input cell number, were 65% for no peptide group and 79% for Hb(64-76) group.

to induction of a profound anergic phenotype upon restimulation with fresh APC and immunogenic peptide (15). We wished to determine if this phenomenon could be extended to T cells of the Th2 phenotype. We have previously reported that 2.102 cells are stimulated to produce IL-4, but not to proliferate, when presented with the APL Asp73 and live APC (34). To explore the consequences of this partial activation, 2.102 cells were stimulated overnight with live APC with or without the Asp73 analog. The T cells were then separated from the APC and restimulated with fresh APC and immunogenic peptide. Whereas T cells previously incubated with APC alone or APC and the nonstimulatory peptide analog Gln72 proliferated vigorously upon restimulation with the immunogenic peptide, 2.102 cells previously incubated with live APC and the Asp73 analog displayed an unresponsive phenotype when challenged with Hb(64-76) (Fig. 3). These data therefore confirm that Th2 cells, which are sensitive to anergy induction by antigen presentation with chemically fixed APC, can be made anergic after partial activation by peptide analog and live APC. This extends our observation of anergy induction with live APC and APL in Th1 cells to include Th2 clones.

It was important to determine if anergy could be extended to other Th2 clones, or if it was a unique property of 2.102 cells. Thus, we examined the response of EW5X, a Th2 clone with identical peptide/MHC specificity as 2.102 but expressing a distinct TCR (Hsu, B.L., and P.M. Allen, unpublished observations). EW5X cells, like 2.102 cells, did not proliferate when presented with antigen by fixed APC while being stimulated to produce IL-4 and IL-3. After such an initial stimulation, EW5X cells displayed an anergic phenotype, being unable to proliferate to live APC and immunogenic peptide (Fig. 2 *C*). Thus, the unresponsive state induced after antigen presentation by chemically fixed APC could be shown for multiple Th2 clones.

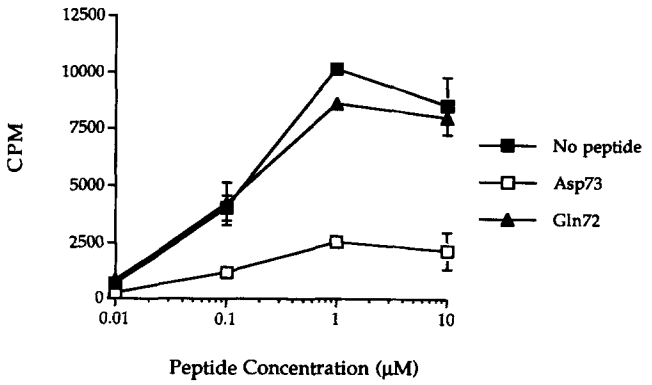


Figure 3. Asp73 APL and live APC induce anergy of 2.102 Th2 cells. 2.102 cells (10^6/well) and mitomycin C–treated CH27 B lymphoma cells (3×10^5/well) were incubated alone or with indicated peptide (100 μM) for 20–24 h. T cells were then separated from APC and challenged in a proliferation assay using 5×10^4 T cells/well, irradiated spleen cells (5×10^5/well), and Hb(64-76) at the indicated concentrations. Cell recoveries after anergy induction, expressed as a percentage of cell input, were 63% for no peptide group, 93.5% for Asp73 group, and 76.5% for Gln72 group. Each point represents the mean of triplicate cultures ± SD. The experiment is a representative of three independent assays.

It was also important to determine if our Th2 clones differ from those of others reported in the literature to be resistant to anergy induction (2), or if our protocol could render such T cells anergic. To address this issue, we examined the tolerance susceptibility of the well-characterized Th2 clone D10 (28) under identical experimental conditions, using the conalbumin immunogenic peptide (residues 132–146) as antigen (21). Like 2.102, D10 cells did not proliferate when presented with antigen by chemically fixed APC (Fig. 4 *A*). Upon analysis of the cytokines produced, it was found that, whereas antigen presented by irradiated APC stimulated significant amounts of IL-3 and IL-4 by D10, antigen presentation by fixed APC failed to stimulate any detectable levels of either cytokine (Fig. 4 *B* and data not shown). Therefore, in contrast to the phenotype observed with 2.102, D10 showed no indication of being partially activated by antigen and fixed APC. Moreover, after such an initial stimulation, D10 cells were able to proliferate normally when restimulated by antigen and irradiated APC, thus failing to display an anergic phenotype (Fig. 4 *C*). These results were consistent with previous literature and led us to conclude that there must be a phenotypic difference between our Th2 clones and D10 that accounts for the difference in tolerance susceptibility.

The Anergic Th2 Cells Can Still Make Cytokines upon Restimulation. The observation that the Th2 clones were able to make cytokines in response to antigen presentation by fixed APC suggested that the signaling requirements for cytokine production and proliferation differ, with a fixation-sensitive component necessary only to activate proliferation. It was of interest to investigate if the anergic Th2 cells could still produce cytokines upon restimulation. To test this, 24-h supernatants were collected from the restimulation assay described in Fig. 2 and analyzed for cytokine production. In contrast to their inability to proliferate upon restimulation, we found that the anergic Th2 cells were still able to produce IL-4 and IL-3, albeit at slightly reduced levels compared with unanergized Th2 cells (Fig. 5, *A* and *B*). These data raise two interesting points. First, the signaling pathway leading to proliferation is distinct from that leading to cytokine production in Th2 cells, with only the proliferation pathway containing a component sensitive to inactivation. Second, anergic Th2 cells, unlike anergic Th1 cells, retain the capacity to make their autocrine growth factor, but have lost the ability to respond to it.

Th2 Anergy is Cyclosporine A Sensitive. Since Th1 anergy can be prevented if cyclosporine A is added with the anergy-inducing stimulus (14, 15), and since Th2 proliferation and IL-4 production can be inhibited by cyclosporine A (35, Sloan-Lancaster, J., unpublished observations), it was of interest to investigate if Th2 anergy was similarly sensitive to inhibition by this drug. 2.102 cells were therefore preincubated with fixed APC and peptide with or without cyclosporine A and then analyzed for their ability to proliferate upon restimulation with fresh APC and antigen. As shown in Fig. 6, the addition of this drug to the preculture prevented the Th2 cells from becoming anergic, indicating that Th2 anergy, like

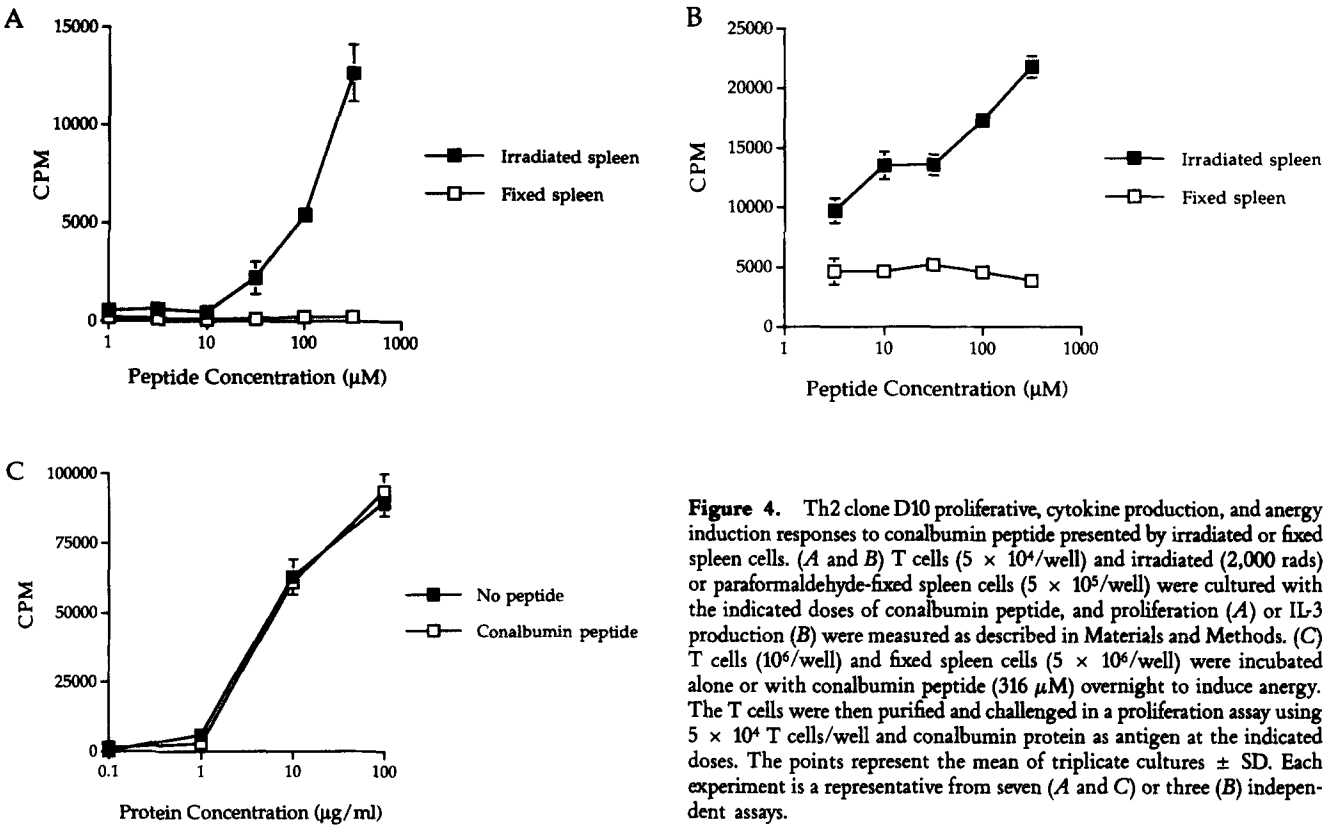
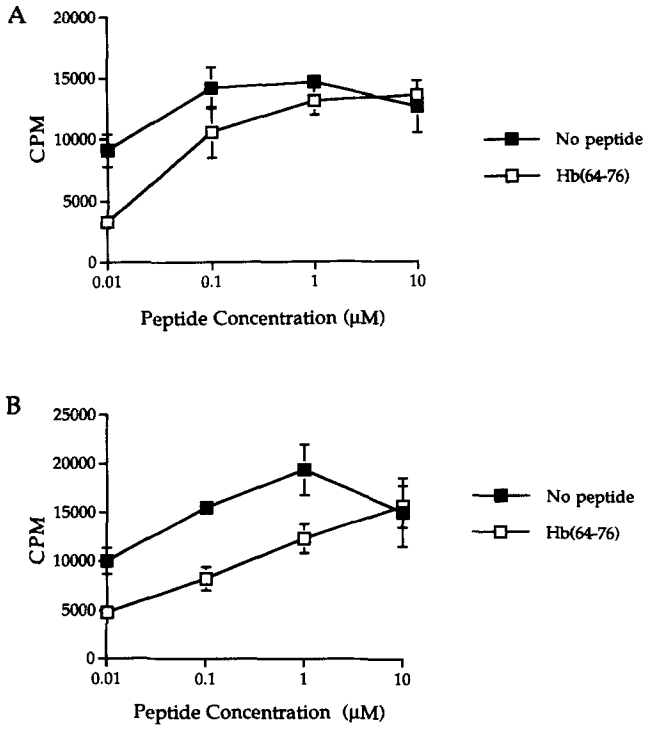


Figure 4. Th2 clone D10 proliferative, cytokine production, and anergy induction responses to conalbumin peptide presented by irradiated or fixed spleen cells. (*A* and *B*) T cells (5×10^4/well) and irradiated (2,000 rads) or paraformaldehyde-fixed spleen cells (5×10^5/well) were cultured with the indicated doses of conalbumin peptide, and proliferation (*A*) or IL-3 production (*B*) were measured as described in Materials and Methods. (*C*) T cells (10^6/well) and fixed spleen cells (5×10^6/well) were incubated alone or with conalbumin peptide (316 μM) overnight to induce anergy. The T cells were then purified and challenged in a proliferation assay using 5×10^4 T cells/well and conalbumin protein as antigen at the indicated doses. The points represent the mean of triplicate cultures ± SD. Each experiment is a representative from seven (*A* and *C*) or three (*B*) independent assays.



Th1 anergy, is cyclosporine A sensitive. This observation suggests that the signaling pathway involved in Th2 cell anergy involves a calcineurin-dependent step.

Addition of Exogenous Costimulation or Growth Factors Does Not Prevent Th2 Clonal Anergy. Following the paradigm of Th1 anergy by fixed APC, we examined the involvement of costimulation in Th2 anergy. IL-1 is well documented as a candidate costimulatory factor for Th2 cells (5–7). To determine if this cytokine was a costimulator for 2.102, the response of these T cells upon antigen presentation by fixed APC was compared with or without the addition of exogenous rIL-1β. Although fixed APC and antigen alone did not

Figure 5. Cytokine production of anergic Th2 cells upon restimulation with antigen. 2.102 cells were stimulated as in Fig. 2 *A*, and supernatants were collected from the challenge assay at 24 h and analyzed for presence of IL-4 (*A*) and IL-3 (*B*) by bioassay using the IL-4–dependent 614 (*A*) or IL-3–dependent GG-1.12 (*B*) cell lines. Cell recoveries after anergy induction stage, expressed as a percentage of input cell number, were 40% for no peptide group and 44% for Hb(64-76) group. The points represent the mean of triplicate cultures ± SD. Each experiment is a representative of three independent assays.

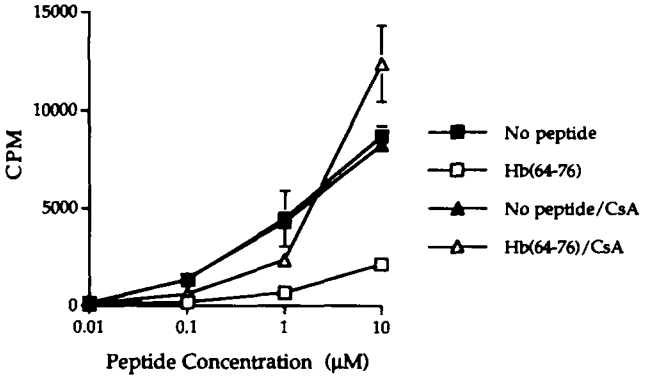
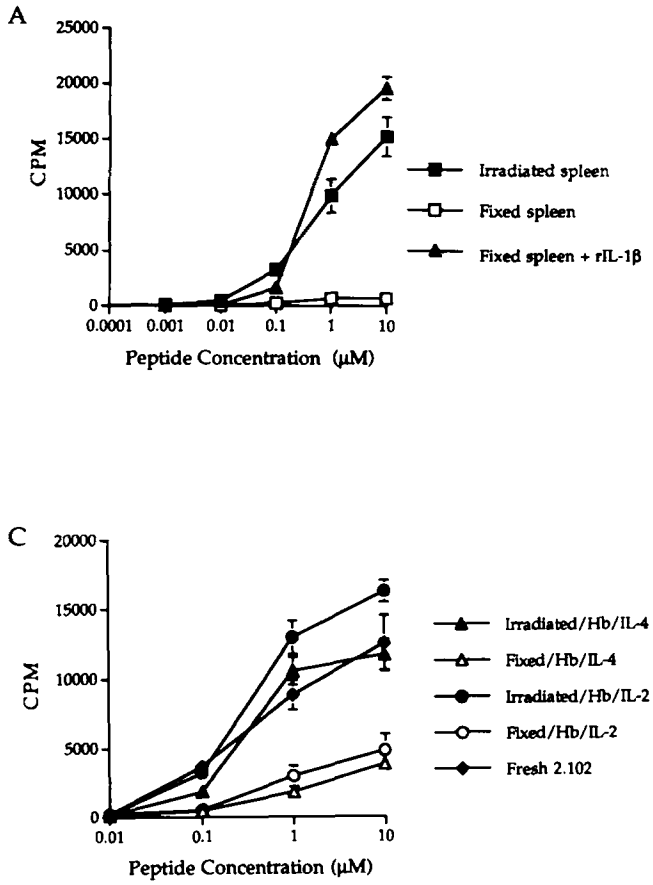


Figure 6. Th2 anergy is prevented by cyclosporine A. 2.102 cells were used in the tolerance assay as in Fig. 2 *A*, with the addition of cyclosporine A (1 μg/ml) to the indicated groups. T cells were then rested for 1 d before being used in the challenge proliferation assay. Cell recoveries after the anergy induction stage, expressed as a percentage of input cell number, were 55.3% for no peptide group, 50% for Hb(64-76) group, 34.2% for no peptide/CsA group, and 47.4% for Hb(64-76)/CsA group. The points represent the mean of triplicate cultures ± SD. The experiment is a representative of four independent assays.

stimulate proliferation, addition of IL-1 to this culture induced a proliferative response from the T cells indistinguishable from that stimulated by antigen and live APC (Fig. 7 *A*). This demonstrated that IL-1 could be used by the Th2 cells as a costimulus, in an analogous manner to the use of CD28 by Th1 cells. However, when the 2.102 cells with fixed APC and antigen plus IL-1 were then challenged with fresh APC and antigen, the T cells still displayed an unresponsive phenotype (Fig. 7 *B*). In contrast, 2.102 cells stimulated with irradiated APC and Hb(64-76) were capable of proliferating vigorously when restimulated with fresh APC and antigen (Fig. 7 *B*). These results led us to conclude that, whereas IL-1 can substitute for the missing factor required for Th2 proliferation in response to antigen presentation by fixed APC, its absence in the initial culture is not responsible for inducing the anergic state. This appears to be in contrast to the role played by CD28 in the Th1 system, where ligation of CD28 is capable both of activating proliferation to antigen presented by fixed APC and preventing anergy induction to such a stimulus (3, Sloan-Lancaster, J., unpublished observations). Thus, it appears that we can induce an anergic state in a Th2 cell when the stimulus it is provided allows the cell to proliferate: i.e., IL-1 as a costimulus is sufficient to restore proliferation to but not to prevent anergy induction by antigen and fixed APC. Whether the anergic Th2 cells can recover from the unresponsive state if allowed to proliferate in the absence of antigen was not addressed in this experiment. We next investigated the effect of adding various growth factors to the primary culture with antigen and fixed APC to assess their effect, if any, on the anergic state. Addition of exogenous IL-2 or IL-4 with antigen and fixed APC did not pre-



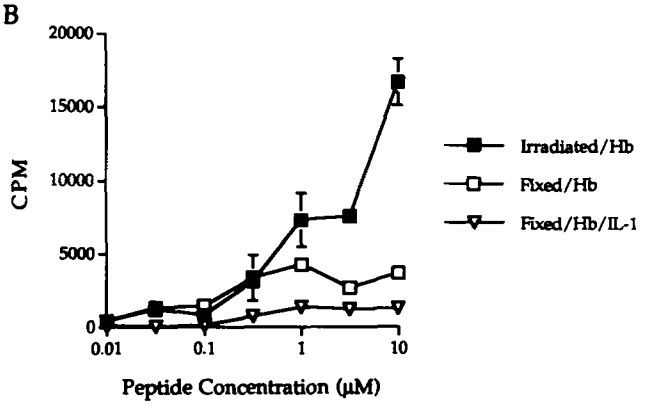


Figure 7. Th2 anergy is not prevented by addition of exogenous costimulators or growth factors. (*A*) Proliferation of 2.102 cells to Hb(64-76) and irradiated or fixed spleen cells ± rIL-1β (50 pg/ml). (*B* and *C*) Anergy induction in the presence of exogenous IL-1 (*B*), IL-2, or IL-4 (*C*). 2.102 cells (2.5×10^5/well) and irradiated spleen cells, fixed spleen cells plus rIL-1β (50 pg/ml), or 2.102 cells (10^6/well) and fixed spleen cells were incubated alone or with peptide (10 μM) for 13 d. Where indicated, either IL-2 or IL-4 (20 U/ml) were included. T cells were then recovered and challenged in a proliferation assay using 5×10^4 T cells/well and Hb(64-76) peptide to stimulate. Fresh unmanipulated T cells were added as a control in *C*. Cell recoveries after the tolerance induction stage, expressed as a percentage of input cell number, were as follows: (*B*) 100% for irradiated/Hb group, 65% for fixed/Hb group and 65% for fixed/Hb/rIL-1β group; (*C*) 54% for fixed/Hb/IL-4 group, 357% for irradiated/Hb/IL-4 group, 91.5% for fixed/Hb/IL-2 group, and 380% for irradiated/Hb/IL-2 group. Each point represents the mean of triplicate (*A*) or duplicate (*B* and *C*) cultures ± SD. Experiments are representatives of >10 (*A*) and 4 (*B* and *C*) independent assays.

vent the resulting unresponsiveness, whereas their addition with live APC and antigen did not diminish the subsequent proliferative response upon restimulation (Fig. 7 *C*).

A study demonstrated that T cells of the Th0 phenotype could be made anergic after TCR engagement in the absence of costimulatory factors (19). The anergic phenotype was characterized by inhibition of IL-2 production and inability to proliferate while still producing IL-4. Since some of the Th0 clones used in the study produced low levels of IL-2, we wanted to rule out the possibility that the 2.102 clone may be making low levels of IL-2. Using three different assays, we convincingly demonstrated that 2.102 T cells do not make IL-2 and are bona fide Th2 cells. First, to examine the growth factor dependence of 2.102, blocking mAbs specific for IL-2 or IL-4 were added to the culture during a proliferation assay.

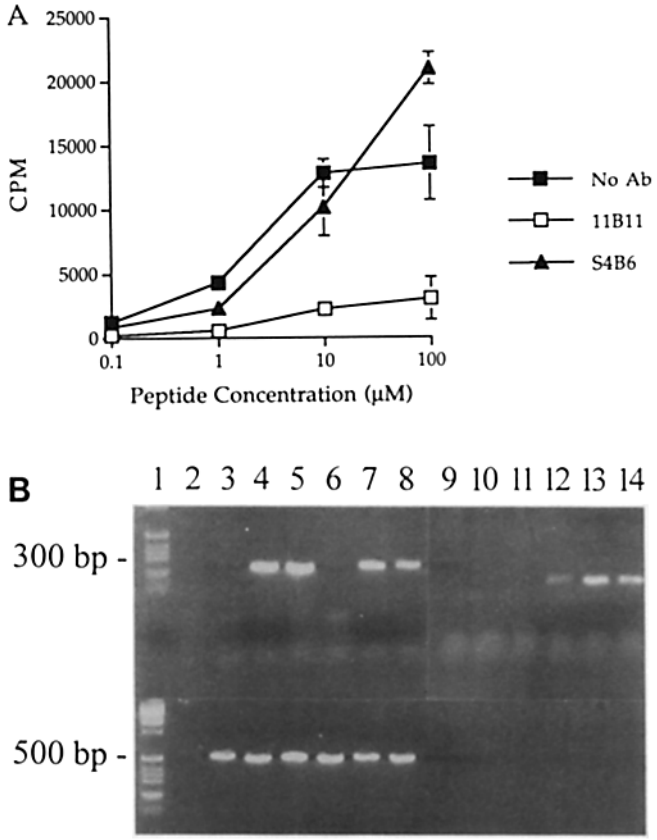


Figure 8. Th2 clone 2.102 does not require IL-2 to proliferate or produce IL-2 upon stimulation. (*A*) Proliferation of 2.102 cells to Hb(64-76) and irradiated spleen cells. Blocking mAbs specific for IL-2 (S4B6, 50 μl/well) or IL-4 (11b11, 25 μl/well) were included in the indicated wells for the duration of the assay. Data points represent the mean of triplicate cultures ± SD. (*B*) Detection by RT-PCR of IL-4 (lanes *3–8*, *upper panel*), IL-2 (lanes *9–14*), or Vβ1 (lanes *3–8*, *lower panel*) mRNA in 2.102 and G2 cells after various stimuli. (Lanes *3* and *9*) unstimulated 2.102; (lanes *4* and *10*) anti-CD3–stimulated 2.102; (lanes *5* and *11*) Hb(64-76)-stimulated 2.102; (lanes *6* and *12*) unstimulated G2; (lanes *7* and *13*) anti-CD3–stimulated G2; (lanes *8* and *14*) Hb(64-76)–stimulated G2. Sizes of amplified PCR products are 188 bp (IL-4), 167 bp (IL-2), and 515 bp (Vβ1). Each experiment is a representative of three (*A*) and five (*B*) independent assays.

Whereas the anti–IL-4 mAb inhibited the proliferation of 2.102 completely, anti–IL-2 mAb failed to diminish the response (Fig. 8 *A*). Second, supernatant from activated 2.102 cells could support the growth of the CTLL cell line, which was completely inhibited by anti–IL-4 mAb (Fig. 1 *B*). Finally, analysis by RT-PCR of the cytokine mRNA present after stimulation of 2.102, with either peptide/APC or anti-CD3 mAb, revealed an activation-dependent synthesis of IL-4 mRNA but no detectable IL-2 mRNA (Fig. 8 *B*). In contrast, the T cell hybridoma G2, made from a fusion of the 2.102 clone, produced detectable IL-2 and IL-4 mRNAs upon activation, consistent with the detection of IL-2 protein from this hybridoma by bioassay (Fig. 8 *B*). Moreover, when the mRNA synthesis pattern of a lysozyme-specific Th0 clone (Yule, T. D., and P. M. Allen, unpublished observations) was analyzed, constitutive IL-4 mRNA and activation-dependent IL-2 mRNA patterns were found (data not shown). In summary, these data confirm that 2.102 is a Th2 clone, since no IL-2 dependence or detection of IL-2 production by these T cells could be demonstrated.

Discussion

This report demonstrates that Th2 clones, similar to Th1 cells, are susceptible to anergy induction in vitro. Specifically, we show that delivery of an initial incomplete signal, which allows cytokine production in the absence of clonal expansion, results in a profoundly tolerant state. The phenotype of tolerant Th2 cells resembles that of anergic Th1 cells in several ways. These include an inability to proliferate in an antigen-specific manner while still able to divide if provided exogenous IL-2 (10, 15), maintenance of the ability to produce cytokines and exert effector functions (2, 36, and Sloan-Lancaster, J., unpublished observations), and susceptibility of the anergy induction pathway to cyclosporine A (14, 15). Furthermore, the anergic phenotype for both T cell subsets appears to be a long-lived state (10, 15). These observations unify Th1 and Th2 cells with regard to anergy and suggest that inhibition of proliferation is the fundamental control mechanism used for anergy for both T cell subsets. This prevention of clonal expansion is therefore an important pathway for controlling amplification of the immune response.

Although there are many similarities in the phenotypes of tolerant Th1 and Th2 cells, there are also some striking differences. First, anergic Th2 cells retain the capacity to make their autocrine growth factor, but they have lost the ability to respond to it. The hallmark of Th1 anergy is that the cells are unable to make IL-2 upon restimulation, yet provision of exogenous IL-2 activates vigorous proliferation (10). This indicates that anergic Th1 cells have normal expression of functional IL-2R, and that their defect is in the ability to synthesize the autocrine growth factor. In contrast, the anergic Th2 cells described here retain the ability to synthesize IL-4, but no longer proliferate to it. This phenotype might be achieved by a downregulation of surface IL-4R or a functional uncoupling of the IL-4R from its intracellular signaling pathway. Alternatively, the inability to proliferate in the presence of sufficient IL-4 may reflect a defect or absence of a

second factor required by the cells to enter the cell cycle. In support of this, several studies have shown that TCR engagement can activate IL-4 production without inducing Th2 cell proliferation (28, 34). The different phenotypes of anergy in the two cell subsets may reflect different stringencies of regulation of the genes encoding their respective growth factors, since several studies indicate that IL-2, but not IL-4, gene activation requires costimulation in addition to TCR engagement (4, 6, 37). Thus, Th2 cells may have evolved a mechanism to become unresponsive that does not involve the inactivation of their autocrine growth factor.

Second, the two Th subsets differ in that the addition of exogenous costimulation does not prevent Th2 anergy induced by chemically fixed APC (10). Although IL-1 functions as a costimulatory factor for some Th2 cells (5–7; Fig. 7 *A*), it had no effect on the anergy induction for 2.102 (Fig. 7 *B*). This observation indicates that exogenous IL-1 is not the natural costimulation utilized by these T cells and ultimately required by them to continually expand. Intriguingly, a recent report has demonstrated that CD28 can function as a costimulator for Th2 clones, presumably by inducing endogenous IL-1 production from the T cells (38). It is thus conceivable that the source of IL-1 in the system may be critical, such that lack of endogenous T cell IL-1 gene activation during TCR engagement leads to subsequent anergy. Although our initial studies indicate that CD28 activation does not prevent anergy of our Th2 cells (Sloan-Lancaster, J., unpublished observations), some other receptor–ligand interaction (e.g., CTLA4-B7-1, -B7-2) may provide such a function to allow endogenous T cell IL-1 production (39–44).

Our previous studies demonstrated that Th1 clones could be made tolerant by presentation of an APL and live APC (15). The phenotype of this tolerant state apparently differs from that induced after antigen presentation with fixed APC, since only in the latter situation can anergy be prevented by addition of exogenous costimulation (14). In this report, we demonstrate Th2 anergy induced both by antigen presentation with fixed APC and by APL presentation with live APC. Although our initial studies indicate that the anergic phenotype resulting from either stimulation appears to be the same, further investigation must be undertaken to assess this. Moreover, the relationship between the signals involved in Th1 and Th2 anergy is unknown at this point.

Several studies have previously demonstrated a failure to tolerize Th2 clones (17, 18). Several potential variables may account for these different experimental findings. These could include the individual antigen specificities, costimulatory dependencies, and level of maturity of the various cells used in the studies. In support of this, Th0 cells are susceptible to tolerance induction, losing the ability to produce IL-2 and proliferate while still able to produce IL-4 (19). The phenotype of these T cells is similar to that of our Th2 clones, in that they lose the ability to respond to IL-4. Moreover, a recent study has demonstrated tolerance induction in vivo, in both the Th1- and Th2-like subpopulations (45). It is argued that the precursors of the Th2 cells, rather than the mature Th2 cells themselves, are susceptible to tolerance induction in this case. It is possible that the Th2 population is less vulnerable to anergy signals than the Th1 population, and that this resistance becomes more prominent as the cells progress in their lineage. One could imagine that established T cell clones can represent cells at different levels of maturity, a reflection of which cell lineage stage the precursor had progressed to when it was cloned. Thus, Th2 clones that are susceptible to tolerance-inducing stimuli, while still exerting a defined Th2 phenotype (Fig. 1 *B* and Fig. 8), would represent cells closer in lineage to Th0 cells than tolerance-resistant Th2 cells (Fig. 4). A comparison of the phenotypes of representatives from the two respective populations could potentially provide insight into this possibility.

If a peripheral tolerance system is designed to deal with self-antigen–specific T cells that have escaped thymic tolerance, all T cell populations should be included for it to be efficient. To date, Th0, Th1, and $CD8^+$ T cells have been shown to become anergic (10, 19, 46). Thus, it is reasonable to assume that Th2 cells would also be susceptible to in vitro tolerance induction. Although unable to proliferate, anergic T cells retain the ability to produce significant levels of cytokines and carry out effector functions upon restimulation. Specifically, anergic Th1, Th0, and Th2 cells still make cytokines (2, 19, and data shown here), whereas anergic Th1 cells and $CD8^+$ T cells retain cytolytic function (36, 46). These observations raise the question of the efficacy, and therefore relevance, of functional anergy in an in vivo setting. However, the immune system depends greatly on clonal expansion for amplification of any response to become effective in a local environment. We propose, therefore, that the ablation of this ability by T cells is a highly efficient way of controlling an autoimmune response in vivo. In support of this, a similar T cell phenotype was found in a system using hen egg white lysozyme (HEL) as a neo-self-antigen. T cell clones generated from HEL-transgenic mice displayed poorer proliferation, but equivalent cytokine production profiles, when compared with T cell clones generated from nontransgenic littermates (47). The transgenic mice showed no signs of autoreactivity, but displayed in vivo tolerance to the neo-self-antigen, demonstrating that limitation of clonal expansion is sufficient for the control of an autoimmune response. In summary, this report demonstrates that Th2 cells, like all other T cell clonal populations, are susceptible to anergy induction in vitro, and implicates an involvement of these cells during control of self-reactive lymphocytes in vivo.

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