Persistence of Peptide-induced CD4$^+$ T cell Anergy In Vitro

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Summary

Clonal T cell unresponsiveness, or anergy, has been proposed as a mechanism of peripheral tolerance in vivo, and as a potential means of curbing unwanted T cell responses. In this study, anergy was induced in a T helper cell (Th) clone reactive to hemoglobin (Hb) peptide 64–76 by coculture of the T cells with live antigen-presenting cells (APCs) and 74L, a peptide analog of Hb(64–76) that contains a single amino acid substitution of leucine for glycine at position 74, or with a low concentration of the agonist ligand. The anergic state was characterized by blunted proliferation and interleukin (IL) 2 production upon restimulation with Hb(64–76), and was not the result of impaired TCR/CD3 downmodulation. The addition of exogenous IL-12 transiently restored proliferation of the anergic lines, but removal of IL-12 from culture returned the T cells to their nonproliferative state. Interestingly, persistence of the anergic phenotype was observed despite biweekly restimulation with antigen, APCs, and IL-2. Thus, T cell unresponsiveness induced by a peptide produced a stable, persistent anergic state in a Th0 clone that was not reversible by stimulation with IL-2 or -12.

Investigation into the process of T cell–mediated immunity increasingly reveals that T cell activation is a multifaceted event, and that differential stimulation of the T cell with various peptide ligands can result in a spectrum of T cell responses ranging from full activation (including proliferation, cytokine production, and effector function) to no activation (1, 2). These T cell responses can be dissociated from one another by modifying the nature and quantity of the TCR ligand, the interaction of costimulatory molecules, and the composition of the local cytokine milieu. Subtle changes in the amino acid sequence of the antigenic peptide can generate altered peptide ligands which can profoundly alter the response of the T cell by coordinating the selective activation of effector responses (3–7).

The importance of costimulatory molecules for T cell responses has been outlined in the two signal model of T cell activation, which states that a signal received by T cells through the TCR/CD3 (signal 1) must be completed by another costimulatory signal (signal 2) for the T cell to be activated. This model arose out of the observation of Jenkins and Schwartz that stimulation of a T cell clone with antigen and chemically fixed APCs not only failed to induce proliferation, but resulted in the further inability of this clone to proliferate when restimulated with antigen and untreated APCs (8). This condition of blunted proliferation that was produced in the T cells is termed clonal unresponsiveness or anergy.

Anergy has been reported for CD8$^+$ and CD4$^+$ T cells, including Th0, Th1, and Th2 clones (9–14). Common methods of inducing anergy include stimulating the T cells with either purified MHC molecules (15), chemically fixed APCs (8), or immobilized anti-CD3 mAb (16), which exclusively trigger TCR/CD3 (i.e., signal 1 only). It has also been observed that partial activation of a Th1 or Th2 clone with live APCs and a peptide analogue can result in anergy (10, 17, 18). Here, we have extended the induction of clonal unresponsiveness by a partial agonist peptide to a murine Th0 clone. Given that costimulation does not prevent or reverse anergy induced by a partial agonist peptide (10, 17), we have addressed the possibility that the peptide initiates an unresponsive state in the T cell that is stably maintained. In fact, in this report we show that the nonproliferative phenotype of the Th0 clone exists long-term in culture and is not reversed by exogenous IL-2 or IL-12.

Materials and Methods

Cells and Reagents. The Th clones were generated from the draining lymph nodes of B10.BR/SGSN (H-2^k) mice (The Jackson Laboratory, Bar Harbor, ME) primed with 200 nM Hb(64–76) (tail and foot pads, subcutaneously in CFA), a synthetic peptide spanning amino acids 64–76 of the β minor chain (*d* allele) of hemoglobin (GKKVITAFNEGLK). Clones were screened for reactivity to Hb(64–76), and the Th phenotype was determined

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based on the production of IL-2, IL-4, and IFN-γ (see Cytokine Measurement, below). T cell clones (2×10^5/well) were restimulated biweekly in a 24-well plate with 5×10^6 spleen cells/well from CBA/JCr mice (National Cancer Institute, Frederick, MD) and 40–50 U of IL-2 obtained from culture supernatants of IL-2-secreting P815 cells (19). Cell culture media consisted of RPMI 1640 supplemented with 1% l-glutamine, 1% Hepes buffer, 100 μg/ml gentamycin (Mediatech, Herndon, VA), 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), and 2×10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO). Peptides were synthesized by FMOC chemistry using a Symphony/Multiplex Peptide Synthesizer and purified by HPLC (Rainin Instruments Co., Woburn, MA).

Cytokine Measurement. IL-2, IL-4, and IFN-γ were measured in culture supernatants of T cells (10^6) stimulated with 5×10^6 γ-irradiated B10.A spleen cells (2,000 rads) and 10 μM Hb(64–76) in a volume totaling 1.5 ml. IL-2 was measured by bioassay using the IL-2–dependent CTLL cell line. 24-h culture supernatants were serially diluted and placed in duplicate in a 96-well plate (100 μl/well) together with 5×10^3 CTLL cells in the presence or absence of 25 μl anti–IL-2 (S4B6) supernatant. The assay was incubated at 37°C for 24 h, then labeled with 0.4 μCi [^{3}H]thymidine (ICN Biomedicals, Irvine, CA). After ~18 h at 37°C, [^{3}H]thymidine incorporation was measured using a Matrix 96 Direct Beta Counter (Packard Instruments, Meriden, CT).

For the measurement of IFN-γ and IL-4, culture supernatants were collected 48 h after primary stimulation as described above, serially diluted, and assayed by antigen capture ELISA using Maxisorp polystyrene 96-well plates (Nunc, Naperville, IL). The IFN-γ capture antibody, R4-6A2, was purified from culture supernatants of cells obtained from the American Type Culture Collection (Rockville, MD). Captured IFN-γ was detected with biotinylated XMG1.2 (PharMingen, San Diego, CA). Samples were quantitated by comparison to a recombinant mouse IFN-γ standard (PharMingen). IL-4 was captured by anti–IL-4 (11B11) antibody purified from culture supernatants. Biotinylated BVD6-24G2 (PharMingen) was used to detect captured IL-4. Recombinant mouse IL-4 was used as a standard (PharMingen).

Proliferation Assay. Clones were cultured at 5×10^4/well in a 96-well flat-bottomed plate (Sarstedt, Newton, NC) together with 5×10^5 γ-irradiated (2,000 rads) B10.A/Cr spleen cells and varying concentrations of peptide. After 48 h of culture, thymidine incorporation was measured by labeling for 18 h with 0.4 μCi [^{3}H]thymidine and by harvesting on a β counter.

Induction of Clonal T Cell Unresponsiveness. Anergy induction performed with modifications according to Sloan-Lancaster et al. (17). 2×10^6 T cells/well were cultured in a 24-well plate with 10^6 DCEK cells (gift from Ron Germain, National Institutes of Health, Bethesda, MD) that had been treated with 50 μg/ml mitomycin C (Sigma Chemical Co.) for 1 h at 37°C. Antigenic peptide was added to the culture at varying concentrations, as noted in the assay. Cultures were incubated at 37°C for 7 d, at which time the T cells were separated from the APC by density gradient centrifugation over a layer of Ficoll-Paque Plus (Pharmacia Biotech, Piscataway, NJ) and restimulated as above.

Assessment of TCR Downmodulation. Assay performed as described by Valitutti et al. (20). In brief, Con A (100 μg) was intraperitoneally injected into a B10.A/Cr mouse to activate macrophages. 3 d later peritoneal exudate cells (PEC) were collected and macrophages were isolated by incubating the PECs in a 96-well plate for 2 h at 37°C. Nonadherent cells were washed away and the remaining macrophages were prepulsed for 2 h with 0, 10, or 100 μM Hb(64–76), washed, and cultured 1:1 with T cell clones (2×10^5). After 7 h, the T cells were stained with anti-CD3 (2C11-145) and an FITC-conjugated secondary anti-mouse IgG mAb (PharMingen). Surface levels of TCR/CD3 were determined by flow cytometric analysis on a FACS®.

Results

Cytokine Profile of Helper T Cell Clone. Helper T cell clones were generated from lymph node cells of H-2^k mice and screened for proliferative responses to Hb(64–76) and production of the cytokines IL-2, IL-4, and IFN-γ. One Th0 clone, designated clone I, was selected for further study based on its reactivity to Hb(64–76) and its elaboration of IL-2, IL-4, and IFN-γ (Fig. 1, Table 1). IL-2 production was detected by bioassay and was confirmed by the addition of neutralizing anti–IL-2 antibody to the assay. In addition, clone I produced IFN-γ (3,230 U/ml) and IL-4 (1,851 pg/ml) as determined by antigen capture ELISA. IL-4 and IFN-γ production was confirmed by reverse transcription PCR, bioassay (IL-4 only), and intracellular cytokine flow cytometry (data not shown). The combined evidence from these assays classifies clone I as a Th0.

A Partial Agonist, 74L, Induces T Cell Unresponsiveness in Clone I. Previous studies have shown that single amino acid changes in the peptide ligand can affect a range of T cell responses such as the level of proliferation, cytokine production, cytolytic function, and B cell help (3, 4). These studies define a partial agonist as a ligand that elicits a submaximal proliferative response but stimulates other T cell effector functions. A weak agonist ultimately reaches the maximal response at higher doses of ligand, but a partial agonist can never approach maximum proliferation. To identify a partial agonist ligand that might anergize clone I, a panel of synthetic hemoglobin analog peptides containing single amino acid substitutions at positions 2, 3, 5, 7, and 8 of the hemoglobin epitope was synthesized. These amino acid positions (69, 70, 72, 74, and 75 of the hemoglobin peptide) have been identified as TCR contact residues by

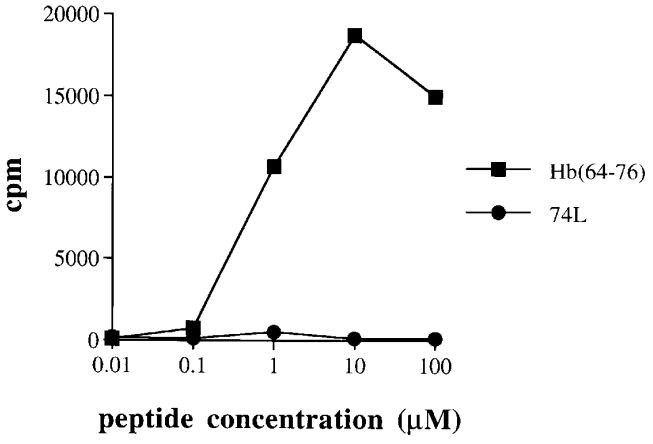

Figure 1. Dose–response of clone I to Hb(64–76) and 74L. Clone I was cultured with APCs and various concentrations of synthetic peptides. Proliferation was measured by incorporation of [^{3}H]thymidine into the DNA of clone I.

Table 1. *Comparison of Preanergic and Postanergic Cytokine Profile*

Clone	Antigen	IL-2*		IFN-γ	IL-4
		Total cpm	α-IL-2 cpm		
				(*U/ml*)	(*pg/ml*)
I	74L	316	40	18	74
I	Hb(64–76)	7,589	2,873	3,230	1,851
I + 10 μM 74L	Hb(64–76)	1,847	781	2,754	288
I + 0.1 μM wt	Hb(64–76)	680	188	1,904	728

*Number of cpm indicates growth of IL-2–dependent cells (CTLL) to culture supernatants, with or without α–IL-2 depletion. IFN-γ and IL-4 levels in culture supernatants were detected by antigen capture ELISA. Culture supernatants were collected from T cells stimulated with syngeneic spleen cells and 10 μM antigen. IL-2 data for clone I stimulated with 74L was taken from a separate assay.

functional data and more recently by the solved crystal structure of hemoglobin bound to IE^k (21, 22). The peptides were then individually screened for their ability to induce the proliferation of clone I, and a range of responses was produced. One peptide, which substitutes a leucine for a glycine at position 74 (74L) of the hemoglobin peptide 64–76 (position 7 of the IE^k-binding epitope), was further examined. In a competitive binding assay, 74L and Hb(64–76) have equal affinities for IE^k (data not shown), and 74L induces little to no proliferation of clone I (Fig. 1). In addition, this analog peptide elicits minimal cytokine production (Table 1), in accordance with previous data in which partial agonist peptides could not separate proliferation from cytokine production by a Th1 (17).

Since 74L induced no proliferation, its ability to functionally activate the T cells was assessed by the induction of anergy. Clone I was incubated with 10 μM 74L peptide and live APC (DCEK). Upon restimulation with Hb(64–76) 1 wk later, clone I demonstrated markedly decreased proliferation compared to control cultures (Fig. 2). The ability of 74L to induce anergy and upregulate CD25 expression (data not shown) indicates that 74L is a partial agonist for clone I. As expected, the culture of clone I with 10 μM Hb(64–76) and live APCs failed to induce unresponsiveness, and anergy of clone I could also be achieved using spleen cells as APCs (data not shown). Therefore, the induction of anergy by 74L is not due to the refractive period which T cells experience after antigen stimulation, nor is it dependent on the APC.

Hb(64–76) Induces Unresponsiveness in Clone I at a Low Concentration. Since partial agonist peptides stimulate minimal proliferation and induce clonal unresponsiveness (10, 17), we examined whether a submitogenic concentration of wild-type (wt)[1] peptide might also induce anergy. A comparison of the proliferation dose–response curves of clone I reveals that the level of proliferation produced by 0.1 μM Hb(64–76) is comparable to the proliferation

[1] *Abbreviation used in this paper:* wt, wild-type.

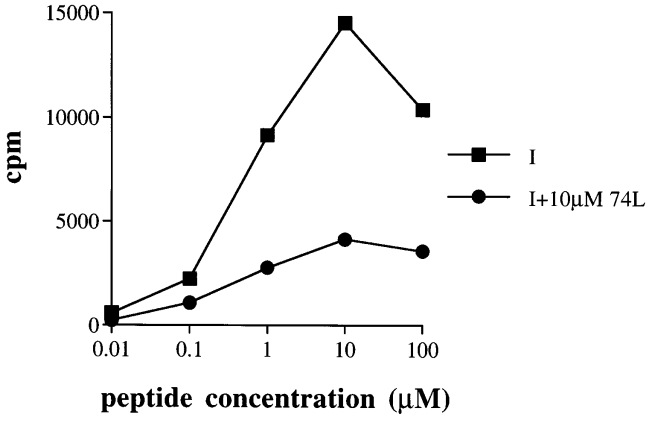

Figure 2. Clone I is rendered unresponsive to Hb(64–76) upon exposure to 74L and live APCs. Clone I was cultured with 10 μM 74L peptide and live DCEK cells. 1 wk later the T cells were isolated and restimulated with APCs and Hb(64–76). Proliferation was measured by DNA incorporation of [^{3}H]thymidine.

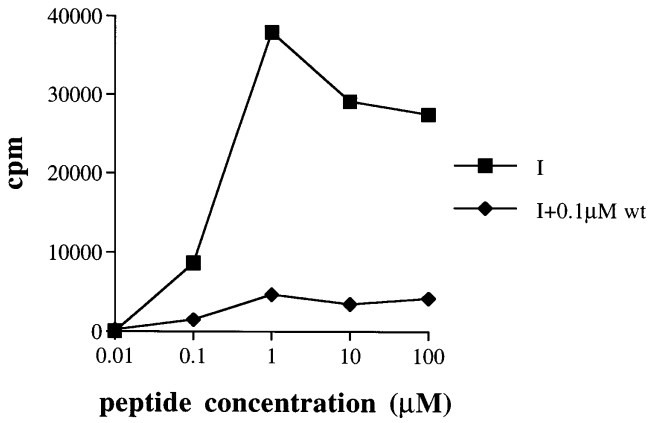

Figure 3. Clone I is anergized by 0.1 μM Hb(64–76) and live APCs. Clone I was cultured with 0.1 μm Hb(64–76) and live DCEK cells, then restimulated 1 wk later with APCs and concentrations of Hb(64–76). Proliferation was measured by DNA incorporation of [^{3}H]thymidine.

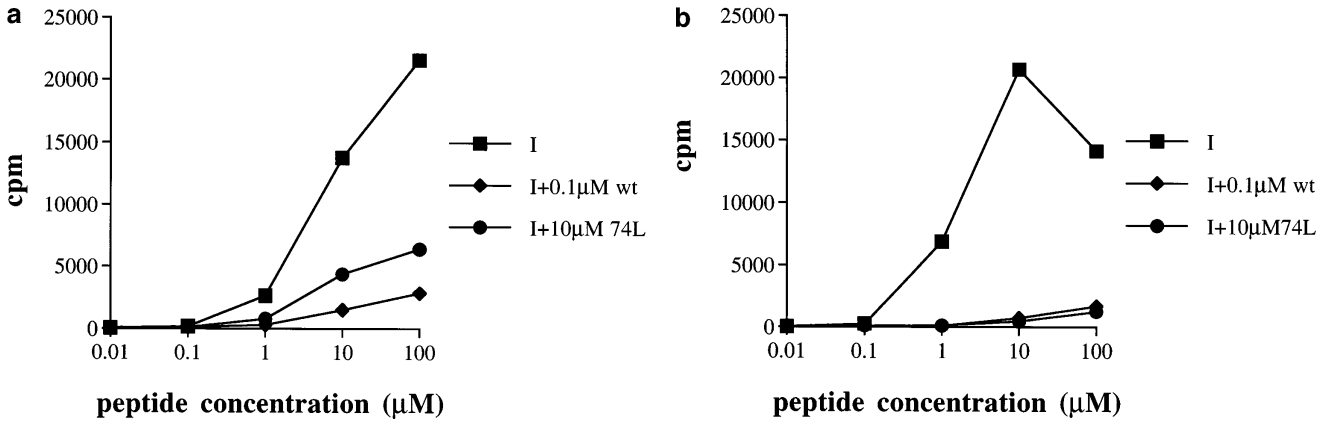

Figure 4. (*a*) The unresponsive clones do not proliferate to Hb(64–76) after one passage in culture. The anergized clones were "passed" once in culture (as described in Materials and Methods) and proliferation was again assessed (as described in Materials and Methods). (*b*) The anergic clones I + 10 μM 74L and I + 0.1 μM wt remain unresponsive to wt antigen after a period of 5 mo in culture. The clones were maintained in culture through a series of biweekly restimulations with antigen and APCs. 5 mo later, the clones were stimulated in vitro with Hb(64–76) and APCs. Proliferation was measured in a proliferation assay, as described.

seen with 10 μM 74L (Fig. 1). T cells were placed in culture with 0.1 μM Hb(64–76) and live APCs, and were tested 1 wk later for their ability to proliferate to Hb(64–76). As shown in Fig. 3, the T cells exposed to 0.1 μM Hb(64–76) were anergic.

Postanergic Cytokine Profile. Anergy is typically accompanied by a loss of IL-2 production (8, 12). Consequently, the cytokine profile of clone I was again determined after a state of unresponsiveness had been established, and negligible levels of IL-2 were produced by the anergic cells (I + 10 μM 74L and I + 0.1 μM wt, Table 1). Significant levels of both IFN-γ and IL-4 were still produced, but the IL-4 was consistently observed at a reduced level relative to clone I. Thus, the anergic Th0 cells selectively lost their ability to produce IL-2.

Persistence of the Anergic Phenotype. It has been proposed that an anergic Th0 would differentiate into a Th2 as a result of the loss of IL-2 (14). To test the postanergic phenotype, the unresponsive Th0 lines were maintained in culture through biweekly restimulation with antigen, APCs, and IL-2. We did not observe a Th2 phenotype, but, intriguingly, the passaged Th0 cells still displayed an anergic phenotype. This was apparent after one cycle in culture (Fig. 4 *A*), and again at the end of a 5-mo period (Fig. 4 *B*), despite the presence of IL-2 (40–50 U), agonist peptide, and competent APCs during restimulation. Anergy was also seen at several intervals leading up to 5 mo (data not shown). Clonal unresponsiveness of the Th0 cells thus persisted through long-term maintenance in culture.

Anergic T Cells Downmodulate the TCR. Several studies have drawn a correlation between the level of $CD4^+$ T cell proliferation and the amount of TCR/CD3 complexes downmodulated from the cell surface in response to peptide–MHC ligand recognition (20, 23). In particular, significant downmodulation occurs at high doses of agonist peptide when the T cells are fully activated, and ligands that only partially activate the T cell cause significantly less downmodu-

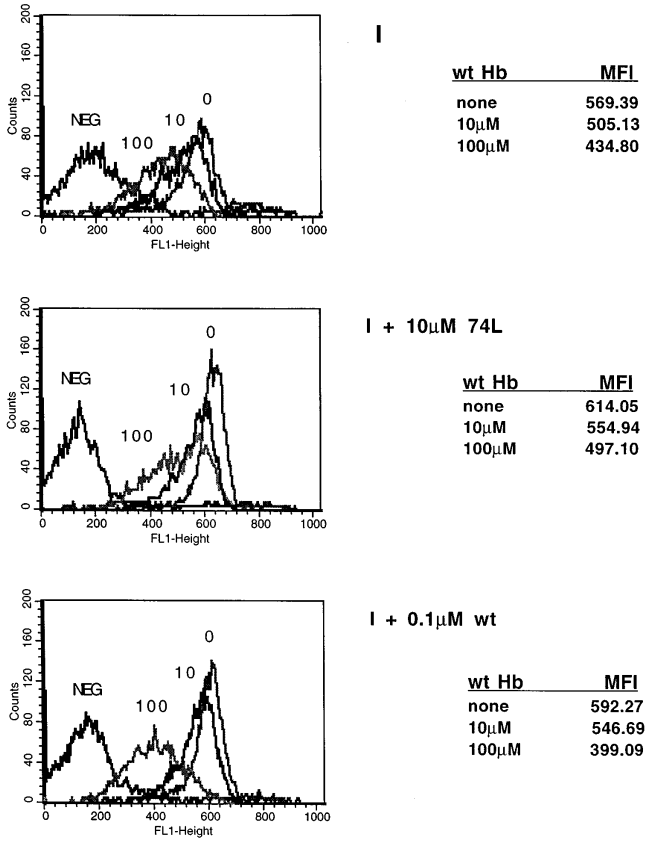

Figure 5. Downmodulation of the TCR in response to antigenic stimulation occurs to equivalent degrees in I and the unresponsive clones. The T cell clones were stimulated for 7 h in vitro with doses of Hb(64–76) and Con A–activated peritoneal macrophages. The cells were then stained with an α-CD3 ab (2C11) labeled with FITC, and surface TCR levels were assessed using flow cytometric analysis.

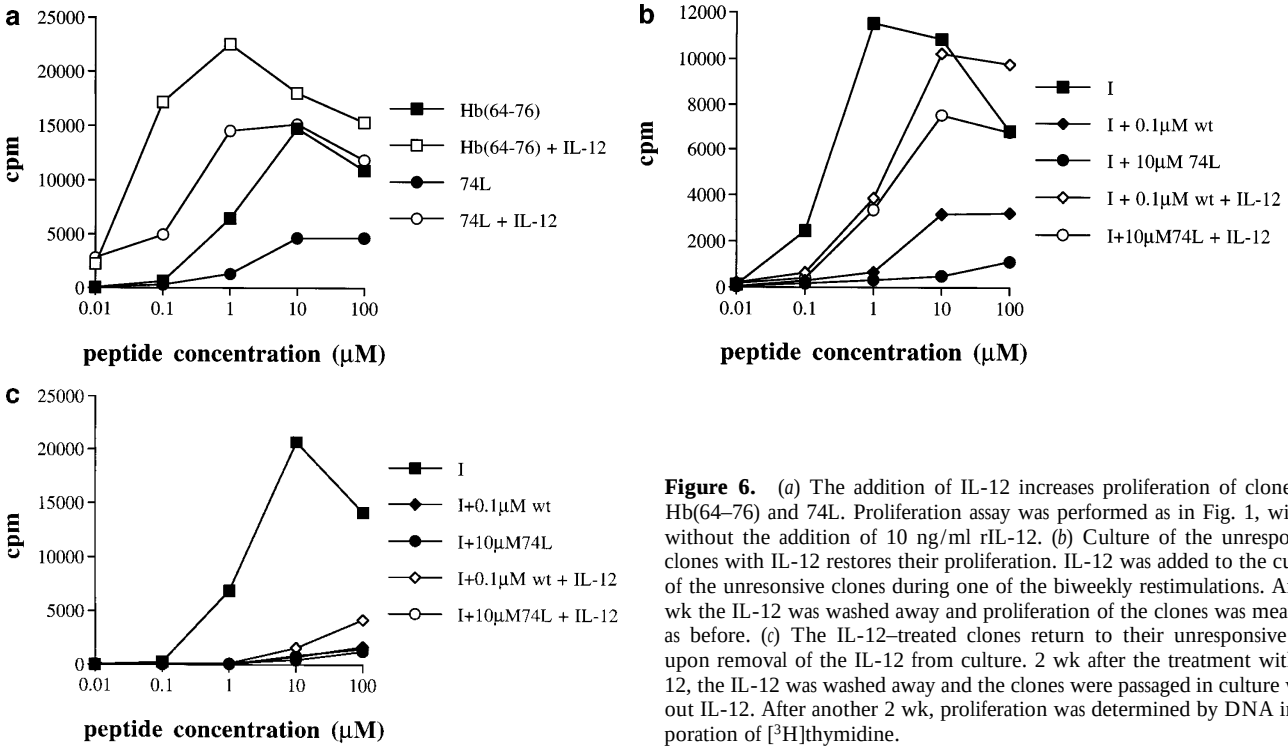

Figure 6. (*a*) The addition of IL-12 increases proliferation of clone I to Hb(64–76) and 74L. Proliferation assay was performed as in Fig. 1, with or without the addition of 10 ng/ml rIL-12. (*b*) Culture of the unresponsive clones with IL-12 restores their proliferation. IL-12 was added to the culture of the unresonsive clones during one of the biweekly restimulations. After 2 wk the IL-12 was washed away and proliferation of the clones was measured as before. (*c*) The IL-12–treated clones return to their unresponsive state upon removal of the IL-12 from culture. 2 wk after the treatment with IL-12, the IL-12 was washed away and the clones were passaged in culture without IL-12. After another 2 wk, proliferation was determined by DNA incorporation of [^{3}H]thymidine.

lation (20). Similarly, our partial agonist, 74L, caused only slight or no TCR downmodulation (data not shown), which correlates with the proliferative response (Fig. 1). To investigate whether the lack of proliferation in the anergic Th0 cells correlated with a failure to downmodulate their TCR/CD3 in response to antigen, flow cytometric analysis was used to assess the relative level of surface TCR/CD3 expression of the anergic cells. Anergic T cells retained an ability to downmodulate their receptors (Fig. 5, *B* and *C*) to a similar degree as the proliferative parent clone I (Fig. 5 *A*). TCR downmodulation peaks at 100 μM because APC were used that were prepulsed with antigen rather than APC that were continually in the presence of antigen, as was the case in Fig. 1.

Exogenous IL-12 Transiently Enhances Proliferation of the Anergic Th0 Cells. The addition of exogenous costimulation has been used to overcome anergy in most of the model systems (9, 24, 25), but costimulation does not alter the anergic phenotype induced with partial agonist peptides (10, 17). Previous work with a Th2 clone demonstrated that a soluble factor (IL-1) could restore the proliferative response to a partial agonist peptide (3), and we have similarly observed that IL-12 boosts the proliferation of clone I to antigen (Fig. 6 *A*). In fact, a significant proliferative response was observed with both agonist peptide (0.1 μM) and partial agonist (10 μM 74L) by the addition of IL-12. To determine the effects of exogenous IL-12 on our anergic Th0 cells, 10 ng/ml recombinant IL-12 was added to the biweekly restimulations, and proliferation to Hb(64–76) was assessed (Fig. 6 *B*). Inclusion of IL-12 in culture with the anergic T cells (but not in the proliferation assay) restored the proliferation of these lines to a similar level as clone I. However, the effect of IL-12 was transient, as the T cells retained their low proliferative response to Hb(64–76) after subsequent passage (antigen, APCs, and IL-2) in the absence of exogenous IL-12 (Fig. 6 *C*). The increased proliferation resulting from IL-12 costimulation was not sufficient to reverse the anergic phenotype.

Discussion

We have demonstrated that a murine Th0 clone can be rendered anergic after exposure to a partial agonist peptide or to a submitogenic concentration of agonist peptide presented by live APCs (Figs. 2 and 3). These results follow from previous studies that demonstrated that partial T cell activation by peptide analogs could lead to anergy of both Th1 and Th2 cells (10, 17), and suggest that CD4$^+$ T cells are anergized by delivering a signal through the TCR under nonproliferative conditions. In our study, nonproliferation correlates with the absence of TCR downmodulation and indicates that the mechanism leading to anergy occurs when T cells interact with ligand that is too weak to induce full T cell activation and TCR internalization. This idea is supported by a recent report in which anergy was induced by a nonmitogenic anti-CD3 mAb that cannot cross-link or aggregate the TCR, but in which intentional cross-linking of the mAb restored a proliferative signal (26). With our Th0 clone, the proliferative responses to partial agonist (74L) or 0.1 μM Hb(64–76) were minimal, yet these ligands signaled through the TCR and drove the T cells into a

state of unresponsiveness. This observation is not unique to clone I since anergy was also achieved using partial agonists (17) or low concentration of agonist (data not shown) with PL.17, a hemoglobin-specific Th1 clone. Because peptide-induced anergy makes no attempt to block the delivery of costimulatory signals, the key factors leading to anergy must be the peptide–MHC ligand itself and the events that it elicits in the T cell upon triggering the TCR.

We report that our anergic T cells are not impaired in the ability to downmodulate TCR in response to antigenic stimulation (Fig. 5), and although proliferation of these cells is severely reduced, maximal downmodulation still correlates with maximal proliferation. These results suggest that surface recognition of peptide–MHC ligand by the T cell occurs properly, and that anergy is likely the result of changes in internal biochemical events. Similar conclusions have been indicated by altered phosphorylation patterns of the TCR/CD3 ζ chain or other proteins after the induction of anergy (27, 28). These results might indicate that anergy can be achieved by multiple routes, each of which diverts the TCR signaling cascade and blocks proliferation. Moreover, the diverted pathway may feed back on itself to maintain the molecular changes that produce anergy, thereby reinforcing the block in proliferation each time the TCR is restimulated with antigen–MHC. Such a system could account for the longevity of the anergic phenotype reported here, and suggests that periodic presentation of antigen by APCs allows T cells to remain in the anergic state. This argument is reminiscent of in vitro B cell studies of Goodnow et al. who observed that functional recovery from tolerance could be demonstrated in the absence of antigen, but that exposure to antigen (lysozyme) in the presence of costimulation (LPS) maintained anergy (29). Similarly, the adoptive transfer of unresponsive lymph node cells to animals lacking the relevant antigen has been reported to result in loss of unresponsiveness (30). The persistence of anergy observed in our study demonstrates that nonproliferative peptides can induce a stable anergic phenotype, which is maintained when restimulated with antigen, IL-2, and costimulation.

T cell anergy has often been characterized by the loss of IL-2 production in addition to nonproliferation (8, 12). This observation prompted Gajewski et al. to suggest that the induction of anergy in a Th0 clone would promote a transition to a Th2 phenotype (14). Our examination of Th0 cells for evidence of a Th2 phenotype are similar to the results of Gajewski et al., in that the T cells lost IL-2 production but continued to secrete IL-4 and IFN-γ (Table 1). The anergic Th0 cells maintained IFN-γ secretion, which is not a characteristic of Th2-like cells, suggesting that IL-2 and IFN-γ production are differentially regulated in Th0 cells. A study using human Th0 cells also noted that analog peptides failed to uncouple IL-4 and IFN-γ production (13).

Another hallmark of Th2 cells as reported by Szabo et al. is the loss of the IL-12 receptor β2 subunit along with the ability to respond to IL-12 (31). Our anergic Th0 cells would be expected to lose IL-12 responsiveness if they in fact shifted to a Th2 phenotype; however, the anergic T cells responded to IL-12 (Fig. 6 *B*). Interestingly, the observation that naive T cells retain IL-12 responsiveness by the addition of IFN-γ during Th2-inducing conditions provides a possible mechanism to explain the continued response to IL-12 and IFN-γ production by our anergic cells (31). Thus, a Th2 phenotype has not been attained, and the induction of anergy in a Th0 clone simply yields an anergic Th0 marked by blunted proliferation and IL-2 production.

Since there was no transition to a true Th2 phenotype after anergy induction, we examined whether the anergic state of the Th0 was itself a stable phenotype that would persist over time despite numerous cell cycles. As seen in Fig. 4, the unresponsiveness produced by peptide antigen persisted through 5 mo of culture consisting of biweekly restimulation. Blunted proliferation was observed after the initial anergy induction, at the 5-mo mark, and at multiple intervening time points. The postanergic cytokine profile helps to explain this observation since in the absence of their primary growth factor, IL-2, the anergic Th0 would still be able to proliferate to IL-4, albeit at a reduced level since IL-4 is a less potent T cell growth factor than is IL-2 (32). It is interesting that a low dose of antigen causes Th cells to differentiate into Th2 cells (33, 34), which raises the possibility that the loss of IL-2 observed with low-dose antigen in our anergic cells is occurring by a similar mechanism.

It is possible that the conditions we have used to induce T cell anergy in vitro correlate to those required for maintaining peripheral tolerance and the control of autoimmune T cells in vivo. We have observed the in vitro induction of T cell unresponsiveness by a peptide analog or a low level of the wild-type antigenic peptide, and the persistence of the anergic state. The relative low abundance of self peptides in vivo (35) would suggest a role for low level agonist or partial agonist in the generation of peripheral tolerance. An autoreactive T cell that escaped deletion in the thymus would be tolerized when it encounters the appropriate self-antigen in the periphery, and once tolerized would be maintained in an unresponsive state. The longevity of anergy is a crucial component of anergy in vivo, since a short-lived anergic cell would simply be another route to death. However, a stable anergic T cell would have the capacity to play a vital role in a productive immune response. For example, anergic T cells that cross-react to a foreign epitope could be stimulated to proliferate by local costimulatory cytokines, such as IL-12, produced during an inflammatory response to a pathogen. These T cells would serve as reinforcement in the clearance of an infection, and then return to an anergic state once the pathogen and associated cytokines were eliminated, explaining the suggested presence of autoreactive lymphocytes in the normal immune response to a pathogen (36, 37). Autoimmune disease would be avoided since the T cells would return to their anergic state after removal of the pathogen and local inflammatory mediators.

In conclusion, anergy can be induced in a $CD4^+$ Th0 clone by nonmitogenic peptide–MHC ligands, whether the

peptide is an analog of the full agonist or is a low concentration of the agonist itself. Furthermore, anergy in Th0 cells persists long-term in culture, indicating that it is a stable T cell phenotype that may correlate with peripheral tolerance in vivo.

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References

1. Evavold, B.D., J. Sloan-Lancaster, and P.M. Allen. 1993. Tickling the TCR: selective T-cell functions stimulated by altered peptide ligands. *Immunol. Today*. 14:602–609.
2. Jameson, S.C., and M.J. Bevan. 1995. T cell receptor antagonists and partial agonists. *Immunity*. 2:1–11.
3. Evavold, B.D., and P.M. Allen. 1991. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science*. 252:1308–1310.
4. Evavold, B.D., J. Sloan-Lancaster, B.L. Hsu, and P.M. Allen. 1993. Separation of T helper 1 clone cytolysis from proliferation and lymphokine production using analog peptides. *J. Immunol*. 150:3131–3140.
5. De Magistris, M.T., J. Alexander, M. Coggeshall, A. Altman, F.C.A. Gaeta, H.M. Grey, and A. Sette. 1992. Antigen analog–major histocompatibility complexes act as antagonists of the T cell receptor. *Cell*. 68:625–634.
6. Windhagen, A., C. Scholz, P. Hollsberg, H. Fukaura, A. Sette, and D.A. Hafler. 1995. Modulation of cytokine patterns of human autoreactive T cell clones by a single amino acid substitution of their peptide ligand. *Immunity*. 2:373–380.
7. Nicholson, L.B., J.M. Greer, R.A. Sobel, M.B. Lees, and V.K. Kuchroo. 1995. An altered peptide ligand mediates immune deviation and prevents autoimmune encephalomyelitis. *Immunity*. 2:397–405.
8. Jenkins, M.K., and R.H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med*. 165:302–319.
9. Schwartz, R.H. 1996. Models of T cell anergy: is there a common molecular mechanism? *J. Exp. Med*. 184:1–8.
10. Sloan-Lancaster, J., B.D. Evavold, and P.M. Allen. 1994. Th2 cell clonal anergy as a consequence of partial activation. *J. Exp. Med*. 180:1195–1205.
11. Otten, G.R., and R.N. Germain. 1991. Split anergy in a $CD8^+$ T cell receptor–dependent cytolysis in the absence of interleukin-2 production. *Science*. 251:1228–1231.
12. Mueller, D.L., and M.J. Jenkins. 1995. Molecular mechanisms underlying functional T-cell unresponsiveness. *Curr. Opin. Immunol*. 7:375–381.
13. Tsitoura, D.C., W. Holter, A. Cerwenka, C.M. Gelder, and J.R. Lamb. 1996. Induction of anergy in human T helper 0 cells by stimulation with altered T cell antigen receptor ligands. *J. Immunol*. 156:2801–2808.
14. Gajewski, T.F., D.W. Lancki, R. Stack, and F.W. Fitch. 1994. "Anergy" of TH0 helper T lymphocytes induces downregulation of TH1 characteristics and a transition to a TH2-like phenotype. *J. Exp. Med*. 179:481–491.
15. Quill, H., and R.H. Schwartz. 1987. Stimulation of normal inducer T cell clones with antigen presented by purified Ia moleules in planar lipid membranes: specific induction of a long-lived state of proliferation nonresponsiveness. *J. Immunol*. 138:3704–3712.
16. Jenkins, M.K., C. Chen, G. Jung, D.L. Mueller, and R.H. Schwartz. 1990. Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. *J. Immunol*. 144:16–22.
17. Sloan-Lancaster, J., B.D. Evavold, and P.M. Allen. 1993. Induction of T-cell anergy by altered T-cell–receptor ligand on live antigen-presenting cells. *Nature*. 363:156–159.
18. Evavold, B.D., J. Sloan-Lancaster, K.J. Wilson, J.B. Rothbard, and P.M. Allen. 1995. Specific T cell recognition of minimally homologous peptides: evidence for multiple endogenous ligands. *Immunity*. 2:655–663.
19. Karasuyama, H., and F. Melchers. 1988. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA. *Eur. J. Immunol*. 18:97–104.
20. Valitutti, S., S. Muller, M. Cella, E. Padovan, and A. Lanzavecchia. 1995. Serial triggering of many T-cell receptors by a few peptide–MHC complexes. *Nature*. 375:148–151.
21. Evavold, B.D., S.G. Williams, B.L. Hsu, S. Buus, and P.M. Allen. 1992. Complete dissection of the Hb(64–76) determinant using T helper 1, T helper 2 clones, and T cell hybridomas. *J. Immunol*. 148:347–353.
22. Fremont, D.H., W.A. Hendrickson, P. Marrack, and J. Kappler. 1996. Structures of an MHC class II molecule with covalently bound single peptides. *Science*. 272:1001–1004.
23. Viola, A., and A. Lanzavecchia. 1996. T cell activation determined by T cell receptor number and tunable thresholds. *Science*. 273:104–106.

24. Harding, F.A., J.G. McArthur, J.A. Gross, D.H. Raulet, and J.P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature*. 356:607–609.
25. Boussiotis, V.A., D.L. Barber, T. Nakarai, G.J. Freeman, J.G. Gribben, G.M. Berstein, A.D. D'Andres, J. Ritz, and L.M. Nadler. 1994. Prevention of T cell anergy by signaling through the gc chain of the IL-2 receptor. *Science*. 266:1039–1042.
26. Smith, J.A., J.Y. Tso, M.R. Clark, M.S. Cole, and J.A. Bluestone. 1997. Nonmitogenic anti-CD3 monoclonal antibodies deliver a partial T cell receptor signal and induce clonal anergy. *J. Exp. Med*. 185:1413–1422.
27. Sloan-Lancaster, J., A.S. Shaw, J.B. Rothbard, and P.M. Allen. 1994. Partial T cell signaling: altered phospho-ζ and lack of zap70 recruitment in APL-induced T cell anergy. *Cell*. 79:913–922.
28. Matsushita, S., and Y. Nishimura. 1997. Partial activation of human T cells by peptide analogs on live APC: induction of clonal anergy associated with protein tyrosine dephosphorylation. *Hum. Immunol*. 53:73–80.
29. Goodnow, C.C., R. Brink, and E. Adams. 1991. Breakdown of self-tolerance in anergic B lymphocytes. *Nature*. 352:532–536.
30. Ramsdell, F., and B.J. Fowlkes. 1992. Maintenance of in vivo tolerance by persistence of antigen. *Science*. 257:1130–1134.
31. Szabo, S.J., A.S. Dighe, U. Gubler, and K.M. Murphy. 1997. Regulation of the interleukin (IL)-12R B2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J. Exp. Med*. 185:817–824.
32. Yokota, T., T. Otsuka, T. Mosmann, J. Banchereau, T. DeFrance, D. Blanchard, J.E. DeVries, F. Lee, and K. Arai. 1986. Isolation and characterization of a human interleukin cDNA clone, homologous to mouse B-cell stimulatory factor 1, that expresses B-cell– and T-cell–stimulating activities. *Proc. Natl. Acad. Sci. USA*. 83:5894–5898.
33. Constant, S., C. Pfeiffer, A. Woodard, T. Pasqualini, and K. Bottomly. 1995. Extent of T cell receptor ligation can determine the functional differentiation of naive $CD4^+$ T cells. *J. Exp. Med*. 182:1591–1596.
34. Hosken, N.A., K. Shibuya, A.W. Heath, K.M. Murphy, and A. O'Garra. 1995. The effect of antigen dose on $CD4^+$ T helper cell phenotype development in a T cell receptor–αβ–transgenic model. *J. Exp. Med*. 182:1579–1584.
35. Rudensky, A.Y., S. Rath, P. Preston-Hurlbert, D.B. Murphy, and C.A. Janeway, Jr. 1991. On the complexity of self. *Nature*. 353:660–662.
36. Janeway, C.A. 1992. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol. Today*. 13:11–16.
37. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu. Rev. Immunol*. 12:991–1045.