

"Anergy" of T_H0 Helper T Lymphocytes Induces Downregulation of T_H1 Characteristics and a Transition to a T_H2-like Phenotype

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Summary

Mature CD4⁺ helper T lymphocytes have been categorized into two major functional phenotypes, T_H1 and T_H2, which produce distinct arrays of lymphokines and which are thought to arise from a pluripotential precursor cell termed T_H0. Clonal anergy can be induced in T_H1 clones by stimulating via the T cell receptor (TCR) complex in the absence of a costimulator molecule; however, anergy has been difficult to demonstrate in T_H2 clones. We show here that treatment of cloned T_H0 lines with anergizing stimuli results in the selective loss of T_H1 characteristics and retention of a T_H2 phenotype. Treated cells exhibit a substantial reduction in interleukin 2 (IL-2) production and antigen-specific cytolytic activity, but retain comparable IL-4 and IL-5 production in response to restimulation via the TCR complex. T_H0 clones exposed to anergizing stimuli also increase in size, thus morphologically resembling T_H2 cells. The signaling characteristics of these cells also are altered, in that they exhibit an elevated basal level of intracellular free calcium which fails to increase significantly with subsequent restimulation, reminiscent of the signaling characteristics of T_H2 cells. "Anergized" T_H0 clones thus share several functional, morphologic, and physiologic properties with cells of the T_H2 phenotype, suggesting that T_H2 cells may arise when T_H0 cells are stimulated via the TCR complex in the absence of a putative costimulator molecule.

Helper T lymphocytes (HTL)¹ perform most of their effector functions via the activity of secreted lymphokines. Cloned CD4⁺ HTL lines can be segregated into at least two mature subsets, termed T_H1 and T_H2, on the basis of the array of secreted lymphokines. T_H1 cells uniquely produce IL-2, lymphotoxin, and IFN- γ , whereas T_H2 cells uniquely produce IL-4, IL-5, IL-6, and IL-10. Cells of both types can produce IL-3, TNF- α , and GM-CSF (1, 2). This distinction based on secreted lymphokines corresponds to functional phenotype. T_H1 cells appear to be responsible for delayed type hypersensitivity reactions and activation of CD8⁺ cytolytic T lymphocytes, whereas T_H2 cells are optimal at providing help for B cell proliferation and Ab production, particularly of the IgE and IgG1 isotypes (3, 4). The T_H1 and T_H2 subsets, therefore, might mediate what have been traditionally referred to as the cellular and humoral classes of immune response, respectively.

Several lines of evidence have suggested that the mature T_H1 and T_H2 cell phenotypes are not present among the population of naive peripheral CD4⁺ lymphocytes, but,

rather, differentiate from that population after a primary activation event. Murine CD4⁺ splenocytes produce predominantly IL-2 but little or no IL-4 or IFN- γ when initially stimulated via the TCR complex (5, 6). However, upon subsequent restimulation, that population secretes lymphokines characteristic of both the T_H1 and T_H2 subsets (5-7). When cloned lines are derived from a bulk population of antigen-primed lymphocytes, three predominant types of cell are obtained: T_H1 cells, T_H2 cells, and a third type, designated T_H0, which continues to produce lymphokines characteristic of both mature subsets (5, 8). The relative proportion of each cell type obtained depends on the exogenous lymphokines present during the activation event or cloning procedure, and probably depends on the type of APC present as well (5, 9-11). Many T_H0 clones, when maintained in culture for several weeks, eventually simplify their lymphokine profile and acquire a more typical T_H1 or T_H2 phenotype (5, 8). Collectively, these observations have suggested a model in which a resting CD4⁺ T cell, upon primary stimulation with specific antigen, differentiates into a pluripotential T_H0 cell. This cell, under the influence of additional factors such as exogenous lymphokines and costimulatory molecules expressed by APCs, differentiates into a mature T_H1 or T_H2 effector

¹ Abbreviations used in this paper: [Ca²⁺]_i, intracellular free calcium; HTL, helper T lymphocyte; PLC, phospholipase C.

cell (12). However, the specific events governing this differentiation process on a clonal level remain undefined.

We have shown previously that T_H1 and T_H2 clones differ with respect to their TCR-associated signal transduction events. The generation of substantial levels of inositol phosphates and the elevation of intracellular free calcium ($[Ca^{2+}]_i$) that are readily detected after stimulation of T_H1 cells via the TCR complex are not observed in T_H2 cells (13). Preliminary results have revealed that this difference may be secondary to differential expression of a particular isoform of phospholipase C (PLC), PLC- $\gamma1$ (Goldfien, R., J. Imboden, T. Gajewski, and D. Qian, unpublished observations). In addition, lymphokine production by T_H1 cells is substantially more sensitive to elevations in intracellular cAMP than is lymphokine production by T_H2 cells (13). The distinct signal transduction characteristics of T_H1 and T_H2 clones probably relate to the unique function of each of the subsets.

Stimulation of T_H1 cells via the TCR complex in the absence of an APC-derived costimulator molecule results, paradoxically, not in activation, but rather in an unresponsive state termed clonal anergy (14–16). This process is analogous to the generation of peripheral tolerance of T lymphocytes that have escaped thymic clonal deletion *in vivo* (17), and is characterized by an inability to secrete IL-2 upon subsequent restimulation. Interestingly, anergy with respect to IL-4 production by T_H2 clones cannot be similarly induced (18), nor is the proliferation of those cells inhibited by anergizing stimuli (19, 20), perhaps as a consequence of their distinct signaling mechanisms. In this study, we have examined the effect of anergizing stimuli on the functional properties and signaling characteristics of T_H0 HTL clones. After induction of anergy, IL-2 production and antigen-specific cytolytic activity of these cells are specifically inhibited, but the ability to secrete IL-4 and IL-5 is preserved, consistent with a functional transition to a T_H2 -like cell. Moreover, these cells increase in size, exhibit increased basal levels of $[Ca^{2+}]_i$, display minimal further increases in $[Ca^{2+}]_i$ after subsequent restimulation, consistent with a transition to a T_H2 -like signaling phenotype. Our observations support a model in which T_H2 cells might arise when a T_H0 intermediate is stimulated via the TCR complex in the absence of a costimulator signal. They also predict that attempts to induce anergy by similar methods *in vivo* may actually generate a T_H2 -driven immune response rather than peripheral tolerance.

Materials and Methods

Animals. Female DBA/2, BALB/c, and CBA/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a barrier facility and used between 6 and 12 wk of age.

Culture Media and Reagents. T cells were grown in culture medium consisting of DMEM plus 5% heat-inactivated FCS, 10 mM 3-(*N*-morpholino)propane sulfonic acid, 5×10^{-5} M 2-ME, 100 U/ml penicillin plus 100 μ g/ml streptomycin, and additional amino acids (21). The CTLL-2 line used for lymphokine assays and the B lymphoma line LK35.2 were grown in the same complete medium containing 10% FCS. OVA, conalbumin, digitonin, Con A, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis,

MO). Ionomycin and Indo-1 acetoxymethylester were purchased from Calbiochem-Novabiochem/Corp. (San Diego, CA).

Recombinant Lymphokines and mAb. Recombinant murine IL-2 and murine IL-4 were kindly provided by the DNAX Research Institute of Molecular and Cellular Biology (Palo Alto, CA). Recombinant human IL-2 was generously provided by Cetus Corp. (Emeryville, CA). Human rIL-2 was used for derivation and maintenance of T cell clones; murine rIL-2 was used as a standard in the IL-2 bioassay. All concentrations are expressed in units per milliliter as defined by the supplier of each of the lymphokines. Hybridoma lines 11B11 (anti-IL-4) (22) and S4B6 (anti-IL-2) (2) were kindly provided by Dr. W. Paul (National Institutes of Health, Bethesda, MD) and Dr. T. Mosmann (DNAX), respectively. The 145-2C11 (anti-CD3- ϵ) hybridoma cell line was kindly provided by Dr. J. Bluestone (University of Chicago) (23).

T Cell Clones. The OVA-reactive T_H0 clones and the T_H1 clone PGL10 were derived from DBA/2 and BALB/c mice using methods described previously (5). The conalbumin-specific T_H2 clone D10.G4.1 was obtained from Dr. C. Janeway (Yale University, New Haven, CT), and has been described (24). Cloned cells were maintained by weekly restimulation with irradiated (2,000 rad) syngeneic spleen cells, antigen, and rIL-2. For experiments, cells were harvested 7–10 d after passage, and purified by centrifugation over a Ficoll-Hypaque gradient (25).

Isolation of Splenic B Cells. Adherent cells were removed by incubating 10^8 spleen cells in tissue culture dishes (model 3003; Falcon Labware, Oxnard, CA) in complete medium containing 5% FCS at 37°C for 1.5 h. Nonadherent cells were recovered by gentle rinsing, and transferred to a glass tube (Vacutainer 6430; Becton Dickinson & Co., Rutherford, NJ) which had previously been autoclaved with 0.2 mg carbonyl iron (9). After incubation at 37°C for 1.5 h, the contents of the tube were gently resuspended and iron was retained in the bottom of the tube with a magnet while the cell suspension was removed with a pipette. Residual iron particles were removed by slow centrifugation (600 rpm for 30 s). T cells and residual macrophages were removed by incubation with the AT83A (anti-Thy1) and M1/70 (anti-MAC-1) mAb along with Low-Tox guinea pig complement (Accurate Chemical & Scientific Corp., Westbury, NY) for 1 h at 37°C. The final cell suspension was purified by Ficoll-Hypaque centrifugation. The resulting cell population typically consisted of >95% B cells as assessed by flow cytometry using anti-Ig and anti-B220 Abs (data not shown).

Induction of Anergy. For treatment with anti-CD3 mAb, culture wells of 24 well plates (Linbro 76-033-055; Flow Laboratories, McLean, VA) were coated overnight at 37°C with 0.5 ml of goat anti-hamster antiserum (Cooper Biomedical, Malvern, PA) at a concentration of 40 μ g/ml in Dulbecco's PBS (DPBS) (26). They were then washed twice with 1 ml DPBS and received 0.5 ml of a 1:20 dilution of 145-2C11 hybridoma supernatant in DPBS and incubated at 37°C for 2 h. After again washing twice, each well received 10^6 cloned T cells in a final volume of 1 ml. For other anergy treatments, cells were stimulated with Ionomycin (1 μ M) or purified B cells (6×10^6) and OVA (800 μ g/ml). Cells were collected 24–72 h later, purified by Ficoll-Hypaque centrifugation, and assayed for functional and biochemical characteristics. Cell yield varied from 40 to 90% of the original number of cells cultured. For lymphokine assays, supernatants were collected after 21–24 h at 37°C, centrifuged to remove cells, and stored at 4°C until assayed.

T Cell Cytolytic Assays. Cloned cells were mixed at the indicated effector/target cell ratios with ^{51}Cr -labeled, OVA-pulsed LK35.2 cells, as described (27). Chromium release was assessed at 5 h.

Lymphokine Assays. IFN- γ concentrations were determined using an ELISA developed by Dr. R. Schreiber (Washington University, St. Louis, MO) (28), who also generously provided the necessary reagents. Activity in culture supernatants was compared to that in a standard (kindly provided by the National Institute of Allergy and Infectious Diseases, No. Gg02-901-533). IL-5 was measured with a specific ELISA, using reagents generously provided by Dr. R. Coffman (DNAX). IL-2 and IL-4 concentrations were determined using a subclone of the CTLL-2 indicator line that proliferated well in response to either lymphokine. Serial dilutions of test supernatants were compared with dilutions of known concentrations of recombinant standards in the presence of anti-IL-2 mAb, anti-IL-4 mAb, both mAbs, or neither. Cell viability was assessed using the colorimetric MTT assay (29).

Analysis of Cells by Flow Cytometry. Cells (2×10^5) purified by Ficoll-Hypaque centrifugation were stained at 4°C with FITC-coupled anti-CD4 (GK1.5), anti-CD3- ϵ (145-2C11), or anti-TCR- α/β (H57-597) mAbs in PBS containing 0.1% BSA and 0.1% sodium azide. Cells were analyzed using a FACScan® (Becton Dickinson & Co., Mountain View, CA); data were collected on 10^4 viable cells and plotted as a histogram of frequency versus fluorescence intensity.

Measurement of Intracellular Free Calcium. Cells were incubated in complete medium containing 5% FCS with 8 μ M Indo-1 acetoxymethyl ester for 1 h at 37°C at a final concentration of 2×10^7 cells/ml. They were then washed with DMEM and incubated for an additional 30–60 min at 37°C in fresh complete medium. Cells were again washed, resuspended at 10^6 cells/ml in complete medium, and maintained in this medium until analysis. For analysis, cells were resuspended in 2 ml of a buffer consisting of 150 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 10 mM glucose, 10 mM MOPS, 0.3% BSA, and 1 mM CaCl₂. Fluorescence measurements were made on samples maintained at 37°C using a spectrofluorometer (model LB-S5; Perkin-Elmer Corp., Norwalk, CT). Indo-1 fluorescence was excited at 330 nm (10-nm bandwidth), and emission was measured at 400 nm (10 nm bandwidth). Con A was used at a final concentration of 10 μ g/ml. Cells were lysed with digitonin to determine F_{max} , and F_{min} was determined by addition of 4 mM EGTA and sufficient Tris base to raise the pH above 8.3. $[Ca^{2+}]_i$ was calculated using the formula: $[Ca^{2+}]_i = K_d[(F - F_{min})/(F_{max} - F)]$, where $K_d = 250$ nM and $F =$ measured fluorescence (30).

Results

Anti-CD3 mAb, Ionomycin, or B Cells Plus Antigen Induce a Subsequent Defect in IL-2 but not IL-4 Production by T_H0 Clones. Unresponsiveness with respect to IL-2 production can be induced in T_H1 clones with several different stimuli that activate the TCR-mediated signaling pathway in the absence of fully competent APCs (16). However, IL-4 production by T_H2 clones is little affected by similar treatments (18, 19, and Gajewski, T., unpublished observations). Since these cell types appear to possess several differences in their TCR-associated signaling mechanisms (13), it was conceivable that the selective effect on IL-2 but not IL-4 production resulted from an anergizing signal being generated in T_H1 but not T_H2 cells rather than the lack of a response of the IL-4 gene to the anergizing stimulus. To address this question, we examined the effect of anergizing stimuli on subsequent responsiveness of T_H0 clones, which produce both IL-2 and IL-4. The cell lines used were believed to be clonal based on lim-

iting dilution frequency analysis, homogeneous morphology, and the fact that they were recloned early after derivation by micromanipulation (data not shown). Five T_H0 clones isolated from BALB/c mice were stimulated for 24 h with immobilized anti-CD3 mAb, purified by Ficoll-Hypaque centrifugation, then restimulated with anti-CD3 mAb for assessment of lymphokine production. Control cells were either harvested directly from passage cultures or incubated overnight in culture medium alone, with similar results. As shown in Fig. 1 A, cells pretreated with immobilized anti-CD3 mAb produced only 0–10% of the amount of IL-2 secreted by control cells. In contrast, IL-4 production was minimally affected, varying from 50 to 200% of control values. No detectable cytokines were produced when anergized T_H0 cells were restimulated with culture medium alone (data not shown), suggesting that IL-4 secretion was not persisting from the initial exposure to anti-CD3 mAb during the in-

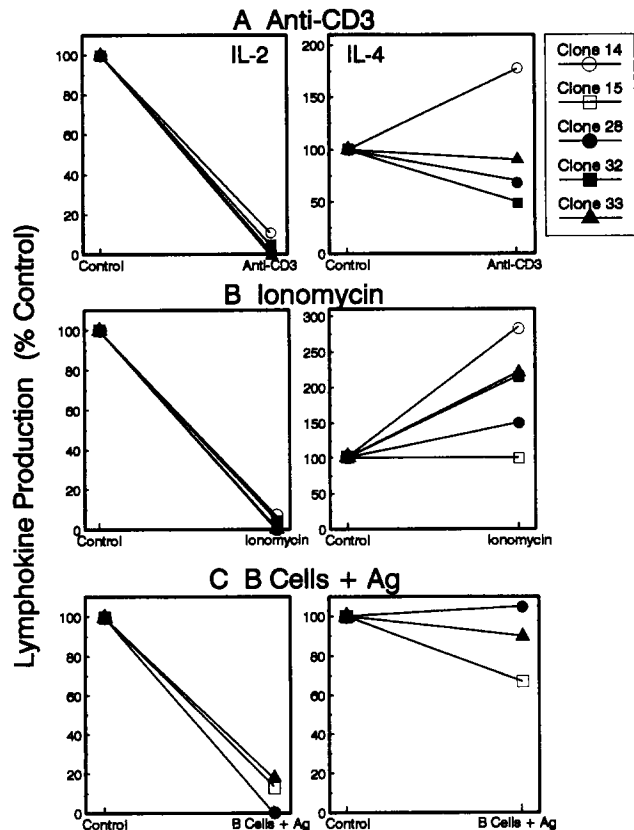


Figure 1. Treatment of T_H0 clones with anergizing stimuli results in a subsequent defect in IL-2 but not IL-4 production. The five T_H0 clones listed (10^6 /well) were stimulated for 24 h with either immobilized anti-CD3 mAb (A), Ionomycin (1 μ M) (B), or syngeneic splenic B cells (6×10^6) and OVA (800 μ g/ml) (C); control cells for each were maintained in rIL-2 (2 U/ml) for the same time period. Cells were then purified by Ficoll-Hypaque centrifugation, restimulated with immobilized anti-CD3 mAb, and supernatants were collected and assessed for IL-2 and IL-4 concentrations. Results are presented as percent control values, and are representative of two to four experiments. Control levels of lymphokines for this experiment (units per milliliter) were as follows: clone 14: IL-2, 200; IL-4, 310,000. Clone 15: IL-2, 7; IL-4, 35,000. Clone 28: IL-2, 460; IL-4, 6,700. Clone 32: IL-2, 1,200; IL-4, 9,300. Clone 33: IL-2, 16; IL-4, 10,000.

duction phase. Similar results were obtained when cells were cultured with anti-CD3 mAb for 72 h or 7 d (data not shown), suggesting that the lack of effect on IL-4 production was not a result of slower kinetics of inhibition of this lymphokine, and also that the inhibition of IL-2 production was not related to a transient unresponsive state occurring during early activation events. In addition, when treated cells were removed from the Ab, rested in culture medium for 7 d, and restimulated with anti-CD3 mAb, they continued to produce dramatically reduced levels of IL-2 compared to control cells (data not shown), arguing that the induced phenotype was relatively long-lived, as has been demonstrated for T_H1 clones (14).

Induction of unresponsiveness to T_H1 cells with respect to IL-2 production appears to be calcium dependent and can be mimicked with the calcium ionophore, Ionomycin (31). It was conceivable that the T_H0 clones generated a sufficient increase in $[Ca^{2+}]_i$ to inhibit subsequent production of IL-2 but not IL-4. Therefore, we examined the effect of Ionomycin pretreatment on subsequent lymphokine production by the T_H0 clones. As shown in Fig. 1 B, Ionomycin also impaired the ability of these cells to produce IL-2 but not IL-4 when restimulated via the TCR complex. In fact, Ionomycin typically enhanced subsequent IL-4 production, exaggerating further the new lymphokine profile produced by the treated cells.

We also determined if T_H0 cells could be anergized with a more physiologic stimulus. It has been shown that anergy can be induced in T_H1 clones with specific peptide presented by chemically treated spleen cells, purified class II MHC molecules in planar membranes, or purified resting B cells (16, 32, 33). We chose purified B cells as APCs for our system because they might represent one cell type that can induce anergy in vivo, and because they effectively stimulate proliferation of T_H2 but not T_H1 clones in vitro (10). As depicted in Fig. 1 C for three T_H0 clones, exposure to antigen and purified B cells also induced subsequent decrease in IL-2 but not IL-4 production. These results suggest that T_H0 cells that encounter B cells presenting the relevant antigenic peptide during an immune response in vivo might subsequently produce IL-4 but substantially less IL-2, effectively transforming the T cell phenotype to a T_H2 -like response.

Effect of Anergizing Stimuli on Production of Additional Lymphokines by T_H0 Clones. Studies of anergy with T_H1 clones have suggested that, whereas IL-2 production and proliferation are dramatically reduced after induction of the anergic state, production of other lymphokines, such as IL-3 and IFN- γ , is marginally affected (16). Therefore, we next examined the effect of anergizing stimuli on the production of additional lymphokines by T_H0 clones. For these studies, a new panel of clones was derived from DBA/2 mice, and screened for the production of IFN- γ in addition to IL-2 and IL-4. As shown in Fig. 2, two subtypes of T_H0 clones were obtained, one which produced IFN- γ and the other which did not. In addition, it was noted that the frequency of IFN- γ -producing T_H0 clones, as well as the concentration of IFN- γ produced by individual clones, was increased if the cells were derived in the presence of exogenous IFN- γ (Fig. 2). T_H0 clones that produced IL-2 and IL-4 were designated T_H0 -A cells, and those that produced IL-2, IL-4, and IFN- γ

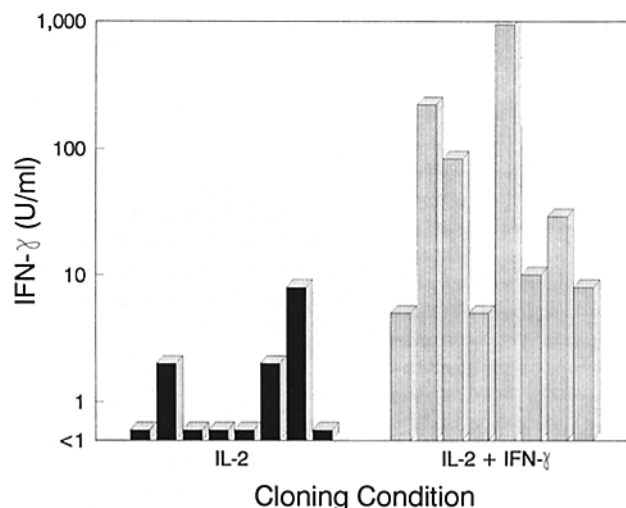


Figure 2. Effect of exogenous IFN- γ on production of IFN- γ by newly derived T_H0 clones. OVA-specific HTL clones were derived in the presence of syngeneic splenocytes, OVA, and either IL-2 (left) or IL-2 and IFN- γ (right) as described previously (5). Eight clones that produced both IL-2 and IL-4 from each group were analyzed for IFN- γ production by specific ELISA after stimulation with anti-CD3 mAb.

were designated T_H0 -B cells, a nomenclature suggested previously (34). All T_H0 clones examined also produced IL-3, IL-5, and TNF activity (data not shown).

T_H0 -A and T_H0 -B clones were treated for 72 h with anti-CD3 mAb, then assessed for the ability to produce multiple lymphokines upon restimulation with anti-CD3 mAb. As shown in Fig. 3 for two T_H0 clones of each subtype, production of IL-2 was completely inhibited by prior exposure to anti-CD3 mAb. In contrast, the ability to produce IL-4, IL-5, and IFN- γ was little affected by the anergizing stimulus. Simi-

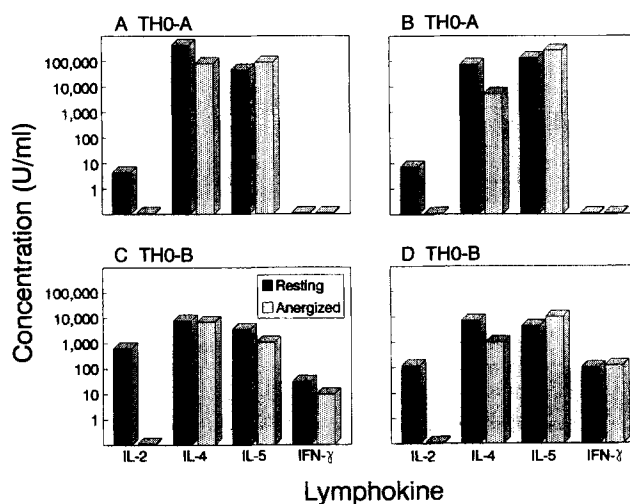


Figure 3. Effect of pretreatment with immobilized anti-CD3 mAb on the subsequent production of additional lymphokines by T_H0 cells. Two representative T_H0 -A and T_H0 -B clones were either harvested from maintenance cultures or anergized for 72 h with anti-CD3 mAb, restimulated with anti-CD3 mAb, and supernatants were assayed for IL-2, IL-4, IL-5, and IFN- γ . Results are representative of two experiments, and similar results were obtained with several additional T_H0 clones.

larly, IL-3 production was only modestly affected by prior exposure to anti-CD3 mAb (data not shown). The T_H0 -A clones that did not produce IFN- γ in the resting state did not acquire the ability to produce IFN- γ after pretreatment with anti-CD3 mAb. Similar results were obtained for several additional T_H0 clones of each subtype (data not shown). These results suggest that the energizing signal exerts a potent inhibitory effect that is relatively specific for production of IL-2. Effects on secretion of additional lymphokines are modest and vary between individual clones. In addition, since the ability to produce both IL-4 and IL-5 persists, they suggest that a functional T_H2 -like phenotype could be retained after exposure of T_H0 -A cells to energizing stimuli.

T_H0 Cells Energized with Anti-CD3 mAb Have Diminished Antigen-specific Cytolytic Activity. Inasmuch as the lymphokine profile of energized T_H0 cells had retained a T_H2 -like pattern, it was of interest to examine other functional procedures as well. The great majority of T_H0 and T_H1 clones derived in our laboratory exhibit substantial antigen-specific cytolytic activity. For T_H0 cells, cytolytic activity usually is greater if the cells produce IFN- γ as well (35, and data not shown). In contrast, many T_H2 clones do not effectively lyse antigen-pulsed targets, particularly clones derived from certain strains of mice, such as BALB/c (35). Interestingly, as depicted in Fig. 4 using two T_H0 clones of each subtype, when T_H0 cells were treated with immobilized anti-CD3 mAb, they subsequently displayed markedly decreased lytic activity for OVA-pulsed B lymphoma cells compared to untreated cells. This effect was nearly complete for the T_H0 -A clones, which generally lysed less well to begin with (Fig. 4, A and B).

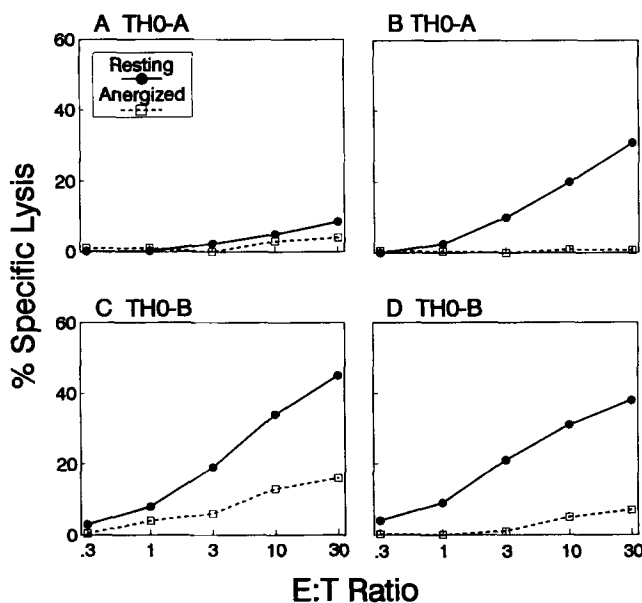


Figure 4. Effect of pretreatment with immobilized anti-CD3 mAb on the subsequent cytolytic activity of T_H0 cells. Two T_H0 -A and T_H0 -B clones were treated for 72 h with immobilized anti-CD3 mAb, collected and purified by Ficoll-Hypaque centrifugation, then tested for the ability to lyse B lymphoma targets that had previously been pulsed with OVA. Results are expressed as percent specific lysis, and are representative of two experiments.

Similar results were obtained with several additional T_H0 clones derived from BALB/c and DBA/2 mice (data not shown). These observations are in stark contrast to those obtained with T_H1 clones, which when energized with anti-CD3 mAb, retained their cytolytic capability (36, and data not shown). These results suggest that exposure of T_H0 clones to energizing stimuli can alter multiple functions in those cells, not only their lymphokine profile. Moreover, they suggest that stimulation of T_H0 cells via the TCR complex might generate a signal that does not occur after stimulation of T_H1 cells, one which results in disengagement of subsequent cytolytic capability.

It has been suggested that T_H1 cells as a group proliferate better than T_H2 cells (37). Hence, the effect of pretreatment of T_H0 clones with anti-CD3 mAb on subsequent antigen-specific proliferation of those cells also was examined. Proliferation of energized T_H0 cells in response to syngeneic spleen cells and OVA was partially reduced, ranging from 10 to 90%, compared to control cells (data not shown). These results also are consistent with inhibition of IL-2 production after exposure to anti-CD3 mAb and predominant utilization of IL-4 rather than IL-2 as an autocrine growth factor.

Surface Phenotype of Energized T_H0 Cells. It was conceivable that treatment of the T cell clones with immobilized anti-CD3 mAb might alter significantly the expression of surface molecules necessary for activation. Inasmuch as restimulation of the cells was performed with immobilized anti-CD3 mAb, expression of the TCR- α/β heterodimer, CD3- ϵ , and CD4 was examined. As shown in Fig. 5, energized T_H0 cells did not express reduced levels of any of these markers,

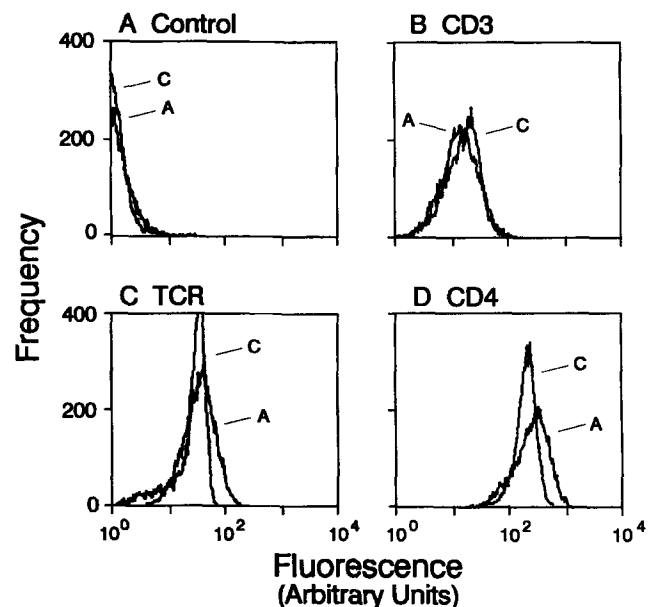


Figure 5. Effect of treatment with immobilized anti-CD3 mAb on expression of cell surface molecules. The T_H0 clone 34 was isolated directly from passage cultures (C), or treated with immobilized anti-CD3 mAb for 72 h (A), then assessed for expression of CD3- ϵ (B), TCR- α/β (C), or CD4 (D) by flow cytometry. Control samples were stained with irrelevant Ab (FITC-OKT3) (A). Results are expressed as relative fluorescence, and are representative of three experiments.

the mean fluorescence of each being comparable to that found in resting cells. The histograms corresponding to the treated cells are slightly more heterogeneous, probably reflecting the relative size increase seen after induction of anergy with anti-CD3 mAb.

The morphologic phenotype of anergic T_H0 cells also was examined. The T_H1 and T_H2 clones we have studied are morphologically dissimilar in that T_H2 cells are typically larger and more rounded compared to T_H1 cells, which are smaller and more club shaped. This is illustrated in Fig. 6 with photographs of a T_H1 clone PGL10 (Fig. 4 A) and the prototypic T_H2 clone D10.G4.1 (Fig. 4 B), purified by Ficoll-Hypaque centrifugation after 7 d in passage culture. The T_H0 clones resembled T_H1 cells, being relatively small and club shaped, although they typically appeared slightly larger than T_H1 cells (Fig. 4 C). Interestingly, after anergy with

anti-CD3 mAb, the T_H0 cells became larger and rounder, appearing more like a T_H2 clone (Fig. 4 D). Similar results were observed after induction of anergy of T_H1 cells (data not shown). After 7 d, they still retained this larger size, as if they had arrested in the middle of blast phase (data not shown). These observations suggest that T_H1 and T_H2 cells might possess different cytoskeletal arrangements, and imply that a reorganization of the cytoskeleton might occur after induction of the anergic state.

Altered Signal Transduction in Anergized T_H0 Cells. We have reported previously that, in contrast to T_H1 clones, T_H2 clones manifest little or no detectable elevation of $[Ca^{2+}]_i$ or inositol phosphates after stimulation via the TCR complex, suggesting utilization of a distinct signal transduction mechanism (13). In light of these results, it was of interest to examine the change in $[Ca^{2+}]_i$ in T_H0 cells, which share char-

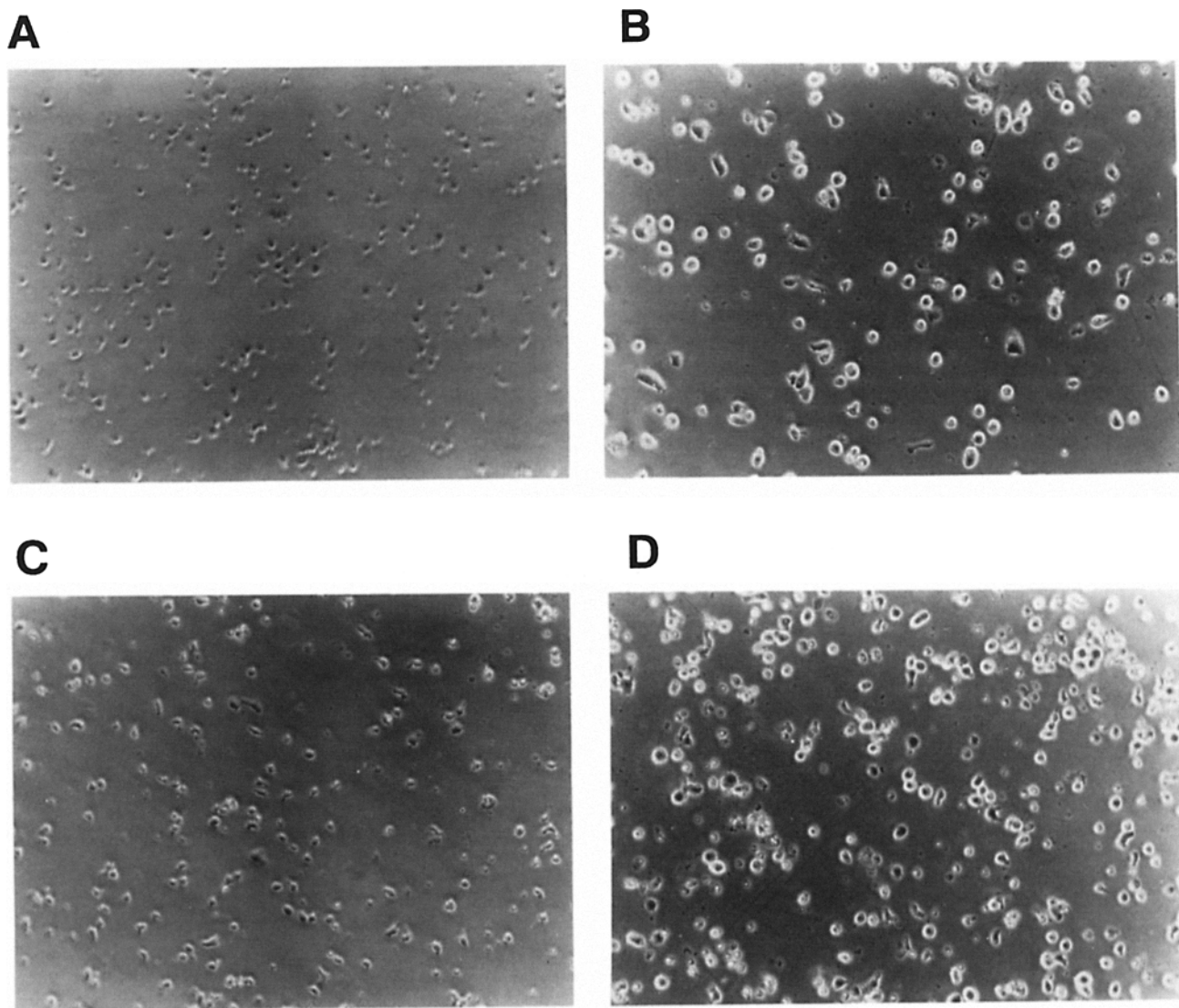


Figure 6. Morphology of T_H0 cells after induction of anergy with immobilized anti-CD3 mAb. The T_H1 clone PGL10 (A), the T_H2 clone D10 (B), the T_H0 clone 34 (C), or the T_H0 clone after treatment with anti-CD3 mAb for 72 h (D) were purified by Ficoll-Hypaque centrifugation. They were then placed back into wells of a 24-well plate (10^6), and photographed with a 35-mm camera fixed to a microscope.

acteristics of both mature subsets. As shown in Fig. 7, after stimulation with Con A, T_H1 cells responded with a 200–300% elevation in $[Ca^{2+}]_i$, whereas T_H2 clones showed a minimal response. Interestingly, the T_H0 clones exhibited an increase in $[Ca^{2+}]_i$ which was intermediate between that seen with T_H1 and T_H2 cells, ranging from 50 to 120% over resting levels. The IFN- γ -producing T_H0 -B clones responded with an approximately twofold greater rise in $[Ca^{2+}]_i$ than the T_H0 -A cells. The presence of a readily detectable calcium elevation in T_H0 clones suggests that they employ, at least in part, a similar signal transduction apparatus to that used in T_H1 cells.

For comparison, change in $[Ca^{2+}]_i$ was also examined in resting splenic $CD4^+$ T cells isolated by positive sorting with anti- $CD4$ mAb (Fig. 7). Resting $CD4^+$ spleen cells, which produce predominantly IL-2 after stimulation via the TCR complex (5), generated a brisk calcium flux in response to Con A, similar to that observed with T_H1 clones. Similar results were obtained using cells enriched by negative depletion with a cocktail of anti- $CD8$, anti-B220, anti-Ig, and anti-MAC-1 mAbs (data not shown), suggesting that the anti- $CD4$ mAb used for sorting had no effect on signaling. If T_H2 cells indeed differentiate from the IL-2-producing population of naive $CD4^+$ T cells, this result suggests that as they acquire the ability to produce IL-4 and pass through a T_H0 intermediate stage, they gradually lose the capability of signaling via the calcium pathway.

It was of interest to explore the effect of anergizing stimuli on signal transduction in T_H0 cells and determine if there were alterations that correlated with the observed changes in function. This was addressed by examining changes in $[Ca^{2+}]_i$ in treated cells. The typical vigorous calcium flux observed with the representative T_H1 clone PGL10 is depicted in Fig. 8 A, and the minimal calcium response observed with the T_H2 clone D10 is represented in Fig. 8 B. Interestingly, the intermediate calcium response seen with

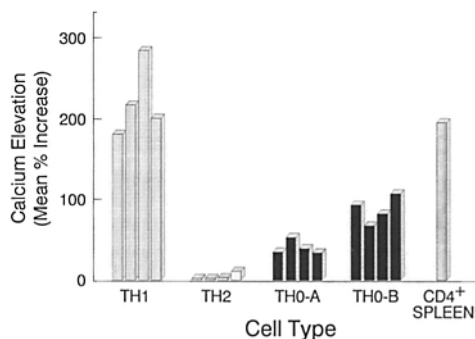


Figure 7. Changes in $[Ca^{2+}]_i$ in response to TCR ligation of HTL subsets. Four HTL clones representing each of the indicated subsets were collected from passage cultures, purified by Ficoll-Hypaque centrifugation, and loaded with the calcium indicator dye Indo-1. $CD4^+$ spleen cells were isolated by positive sorting with FITC-conjugated anti- $CD4$ mAb and also loaded with Indo-1. They were then stimulated with Con A ($10 \mu\text{g}/\text{ml}$), and changes in fluorescence were monitored using a spectrofluorometer. Calcium concentrations were determined as described in Materials and Methods. Results are expressed as percent increase over baseline values.

a representative T_H0 -B clone (Fig. 8 C) was nearly completely abrogated after induction of anergy with anti- $CD3$ mAb (Fig. 8 D). Thus, anergic T_H0 cells have a blunted elevation of $[Ca^{2+}]_i$ upon restimulation, similar to that observed with T_H2 clones.

This response was examined more carefully with several additional T_H0 -A and T_H0 -B clones. When actual $[Ca^{2+}]_i$ concentrations were calculated, several conclusions emerged. First, the IFN- γ -producing T_H0 -B cells had a lower basal $[Ca^{2+}]_i$ compared to the T_H0 -A clones (Fig. 9). This result is reminiscent of observations made with T_H1 and T_H2 clones, the former having relatively lower $[Ca^{2+}]_i$ in the resting state compared to the latter (13). Second, after primary activation via the TCR complex, the $[Ca^{2+}]_i$ for all clones approached approximately the same plateau, around 200 nM in these experiments. Third, after anergy with anti- $CD3$ mAb, the basal $[Ca^{2+}]_i$ was markedly elevated at ~ 200 nM, and did not significantly increase after subsequent restimulation via the TCR complex (Fig. 9). This signal phenotype is similar to that observed previously with T_H2 clones (13). Collectively, these results suggest that as HTL develop down the T_H2 pathway, they acquire an increased basal intracellular calcium concentration, which is not increased much further when those cells encounter TCR stimulation.

Discussion

Clonal anergy was first described in a T_H1 clone after stimulation with chemically treated spleen cells and specific antigenic peptide (14). Subsequently, it was found that anergy could be induced by additional stimuli, including antigenic

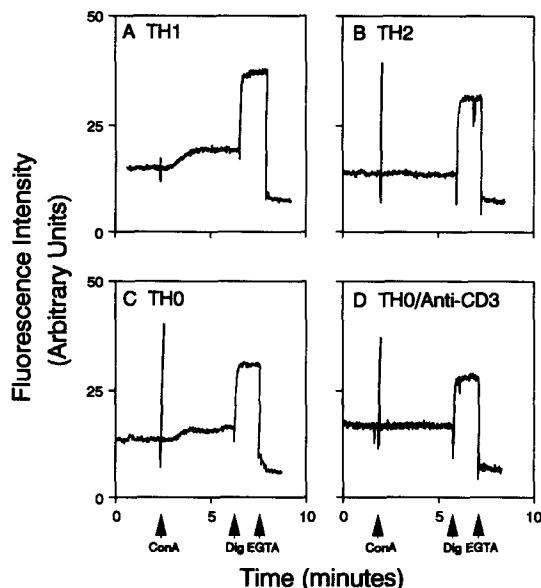


Figure 8. Anergized T_H0 cells have a diminished calcium response. The T_H1 clone PGL10 (A), the T_H2 clone D10 (B), the T_H0 clone 34 isolated from passage culture (C), and the T_H0 clone after pretreatment with immobilized anti- $CD3$ mAb (D) were purified by Ficoll-Hypaque centrifugation. They were then loaded with Indo-1, and calcium changes were monitored in response to Con A ($10 \mu\text{g}/\text{ml}$) as in Fig. 7. Results are representative of three experiments.

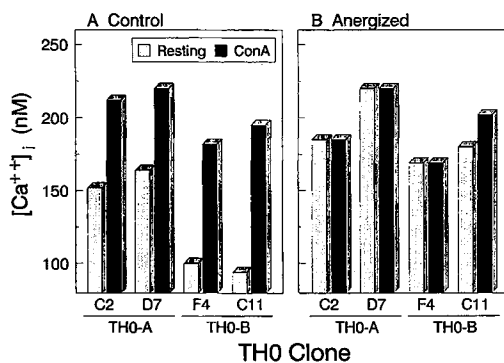


Figure 9. Basal and activated $[Ca^{2+}]_i$ levels in T_H0 clones before and after induction of anergy. Two T_H0 -A and two T_H0 -B clones were either harvested from maintenance cultures (Control) or anergized for 72 h with anti-CD3 mAb (Anergized). Calcium concentrations were calculated in this basal state and after stimulation with Con A ($10 \mu\text{g/ml}$) for 3 min. Results are representative of at least two experiments.

peptide presented either by purified class II MHC proteins in planar lipid membranes or by small resting B cells, anti-TCR mAbs, the T cell mitogen Con A, and the calcium ionophore, Ionomycin (15, 16, 31–33). All these stimuli lack the ability to provide a costimulator signal delivered by particular APCs that prevents induction of anergy. This cofactor may be identical to the cofactor necessary for optimal proliferation of T_H1 cells (10). In fact, it has been suggested that proliferation per se is the event necessary to avoid induction of anergy (38). The fact that Ionomycin alone is sufficient for the generation of the unresponsive state suggests that calcium elevation in the absence of a signal delivered by the costimulator molecule is the essential biochemical step during the induction phase.

In contrast to T_H1 cells, anergy of T_H2 cells with respect to IL-4 production has been difficult to demonstrate (18). It was conceivable that this difference was secondary to different signals being generated after engagement of the TCR complex in the two cell types. In our present study, immobilized anti-CD3 mAb, Ionomycin, or purified B cells and antigen, all were able to induce anergy with respect to IL-2 but not IL-4 production in T_H0 clones. The fact that anergizing stimuli led to inhibition of expression of one lymphokine gene but not another in the same cells, suggests that the anergy effect is specific for IL-2 and not IL-4 gene expression. It is likely that IL-4 production by T_H2 clones cannot be anergized because expression of the IL-4 gene is insensitive to the effects of anergizing stimuli. In fact, Ionomycin alone actually stimulates production of IL-4 by T_H2 clones (13). Other lymphokines that are only partially inhibited after exposure to anergizing stimuli, such as IFN- γ and IL-3 (16), probably rely on multiple signaling pathways for their expression, only some of which are inhibited in the anergic state.

Recently, other investigators (34) have reported similar induction of anergy by anti-CD3 mAb with respect to IL-2 but not IL-4 production in T_H0 clones. In that study, it is argued that the cells do not acquire a T_H2 phenotype because the anergizing stimulus also induces reduced ability to proliferate in response to IL-4. However, T_H2 cells generally

proliferate less vigorously in response to antigenic stimulation than T_H1 cells. In addition, we have observed that the majority of our clones previously characterized as T_H2 cells do not proliferate well in response to exogenous IL-4, although their modest autocrine proliferation is inhibited by anti-IL-4 mAb (Gajewski, T., unpublished observations). Therefore, the reduced ability to proliferate might not be uncharacteristic of T_H2 clones, and is not inconsistent with the possibility that induction of anergy in T_H0 cells may lead to a T_H2 -like phenotype.

Antigen-specific cytolytic activity also was inhibited in anergic T_H0 cells, demonstrating that more than one functional trait can be significantly affected. Interestingly, cytolytic activity was not affected after anergy of several T_H1 clones (36, and Lancki, D. W., and T. Gajewski, unpublished observations), nor was it found to be inhibited in anergic IL-2-producing $CD8^+$ CTL clones (40). These results suggest that the TCR-mediated signals that are coupled to the cytolytic machinery in T_H1 and T_H0 cells may be differentially influenced by anergizing stimuli. Alternatively, T_H1 and T_H0 cells might employ distinct mechanisms of cytotoxicity (27), only one of which is affected in the anergic state. The fact that multiple T_H1 -like functions were reduced after induction of anergy of T_H0 cells supports the notion that a T_H2 -like phenotype is emerging under those conditions. Whether or not anergy of T_H0 cells renders them better able to provide help for B cell proliferation and Ab production remains to be examined.

Anergized T_H0 cells exhibit an elevated basal level of $[Ca^{2+}]_i$ and a blunted calcium flux upon restimulation, signaling attributes also possessed by T_H2 cells. Preliminary results have suggested that the diminished elevation of $[Ca^{2+}]_i$ observed in T_H2 clones, as well as anergized T_H0 clones, may correlate with decreased detectable PLC- γ 1 expression (Goldfien, R., J. Imboden, T. Gajewski, and D. Qian, unpublished observations). In addition, the pattern of tyrosine phosphorylated substrates seen after TCR ligation of anergized T_H0 clones also is altered, more resembling that of T_H2 cells (Gajewski, T., and D. Qian, unpublished observations), implying that multiple aspects of TCR-mediated signal transduction are modified in anergized T_H0 clones. If T_H2 cells indeed arise when T_H0 cells are exposed to anergizing stimuli, these results also suggest the possibility that the signaling characteristics of T_H2 cells may be a secondary event, the result of repeated engagement with APCs lacking costimulator activity, induction of anergy with respect to IL-2 production, and downregulation of the T_H1 -type signaling pathway. Thus, the lack of a calcium flux may correlate with the T_H2 phenotype, essentially being a marker for their history of being activated in the absence of a costimulator signal, but might not actually mediate the T_H2 phenotype.

Several APC-derived costimulator molecules and their associated receptors have been identified that may mediate costimulation and prevent induction of anergy in T_H1 cells. CTLA4 (41) and CD28 (42) are two costimulator receptors expressed on T lymphocytes, both of which can bind to the molecule B7 expressed on activated B lymphocytes and other cell types (43). Heat stable antigen also has been suggested

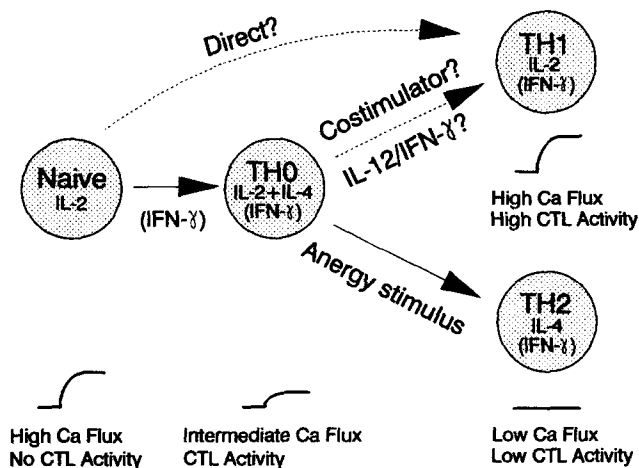


Figure 10. Model for the ontogeny of T_H1 and T_H2 cells based on signaling differences and the concept of anergy. Our results are consistent with a model in which resting naive $CD4^+$ T cells exhibit a brisk calcium flux and produce predominantly IL-2 upon primary stimulation. Primary activation gives rise to the T_H0 phenotype, which has an intermediate calcium flux and produces lymphokines characteristic of both T_H1 and T_H2 cells. In the presence of anergizing conditions, this cell loses its T_H1 attributes and retains characteristics of a T_H2 cell. The fact that both resting $CD4^+$ splenic T cells and T_H1 cells exhibit a vigorous calcium flux in response to TCR ligation suggests that the T_H1 differentiation pathway might proceed independently of the T_H0 intermediate. Stimuli inducing differentiation of T_H1 cells from T_H0 cells are suggested by other studies, but remain speculative on the clonal level.

to serve as a receptor for a costimulator molecule (44). It will be of interest to examine the expression and activity of these molecules with respect to the various HTL subsets, and to determine if simultaneous ligation of any of these molecules and the TCR complex can prevent the transition of T_H0 cells into a T_H2 phenotype.

Several cytokines have been shown to facilitate differentiation of newly activated HTLs into mature T_H1 or T_H2 cells. IFN- γ appears to favor emergence of a T_H1 phenotype (5), perhaps by increasing the frequency of T_H0 cells that produce IFN- γ (see Fig. 2). IL-12 has recently been shown also to promote generation of a T_H1 phenotype, by a mechanism that may depend upon IFN- γ activity (45). Conversely, the presence of IL-10 and IL-4 during HTL activation appears to result in preferential emergence of a T_H2 phenotype (46). It is unclear whether these cytokines act directly on T lymphocytes, or indirectly via APCs, to exert all of their differentiation-inducing effects. It is conceivable that expression of costimulator molecules on the APC population, or responsiveness of the T cells to costimulation, is modulated by these cytokines, thus determining if the developing T cell is exposed to stimulation via the TCR alone or simultaneously with a costimulatory signal. These possibilities remain to be examined.

A proposed model for the ontogeny of T_H1 and T_H2 cells with the potential influence of anergizing stimuli is summa-

rized in Fig. 10. Naive $CD4^+$ T cells, when first activated via the TCR complex, respond with a brisk elevation in $[Ca^{2+}]_i$ and produce predominantly IL-2. This primary activation event induces a differentiation process that allows the cell to express additional lymphokine genes, perhaps all lymphokine genes that HTL are capable of transcribing, thus giving rise to the T_H0 phenotype. The presence of IFN- γ during this period may determine if the T_H0 cell itself acquires the ability to produce IFN- γ . Associated with an expanded lymphokine program is cytolytic activity, and moderately altered signal transduction, manifested by a reduced calcium flux. It is possible that the T_H0 cell expresses quantitatively less of the T_H1 signaling enzymes, or that acquisition of T_H2 traits inhibits some aspects of T_H1 signaling. When cells at the T_H0 stage are stimulated in the absence of a costimulator molecule, the T_H1 program might be inhibited, giving rise to a T_H2 phenotype. These cells then have reduced cytolytic capability and lack a substantial calcium flux. In addition, the basal level of $[Ca^{2+}]_i$ rises as the developing cell becomes more T_H2 -like. Such a situation might occur when resting B cells are the predominant APCs participating in the ongoing immune response, as these cells can induce anergy of T_H1 clones (32, 33) and inhibit the T_H1 characteristics of T_H0 clones (see Fig. 1). Mature T_H2 cells, therefore, retain the ability to proliferate in the absence of costimulator signals, and B cells can serve as optimal APCs for this cell subset (10). Conversely, although not examined in this study, cells of the T_H1 phenotype may arise when the T_H0 cells are stimulated in the presence of a costimulator signal, perhaps under the additional influence of the cytokines IL-12 and IL- γ . However, it is interesting that both naive HTL and mature T_H1 cells exhibit a vigorous calcium flux, whereas T_H0 cells have one of intermediate magnitude. This result suggests the possibility that T_H1 cells may arise directly from naive $CD4^+$ HTL precursors, and that the T_H0 cell is only an intermediate in the differentiation pathway of T_H2 cells. These aspects of the ontogenetic model have yet to be examined.

The cell culture model for inducing clonal anergy probably reflects a mechanism by which peripheral tolerance of T lymphocytes can be achieved in vivo (17, 47). Application of these concepts might provide a means by which to deliberately induce specific T cell unresponsiveness during situations of undesirable T cell activation, such as autoimmune diseases or allogeneic transplant. However, our results suggest that attempts at inducing specific anergy in vivo, after an immune response has already been initiated and a T_H0 phenotype has been generated, might result not in complete anergy, but rather a T_H2 -centered immune response. In fact, it has been demonstrated that neonatal exposure to alloantigens results, not in complete tolerance, but in the generation of cells that produce IL-4 (48). Additional in vivo models will need to be explored to further test these hypotheses.

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