“Anergy” of Tn0 Helper T Lymphocytes Induces Downregulation of Tn1 Characteristics and a Transition to a Tn2-like Phenotype

By Thomas F. Gajewski, David W. Lancki, Risa Stack, and Frank W. Fitch

From The Committee on Immunology, Department of Pathology, and the Ben May Institute, the University of Chicago, Chicago, Illinois 60637

Summary

Mature CD4+ helper T lymphocytes have been categorized into two major functional phenotypes, Tn1 and Tn2, which produce distinct arrays of lymphokines and which are thought to arise from a pluripotent precursor cell termed Tn0. Clonal anergy can be induced in Tn1 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 Tn2 clones. We show here that treatment of cloned Tn0 lines with anergizing stimuli results in the selective loss of Tn1 characteristics and retention of a Tn2 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. Tn0 clones exposed to anergizing stimuli also increase in size, thus morphologically resembling Tn2 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 Tn2 cells. “Anergized” Tn0 clones thus share several functional, morphologic, and physiologic properties with cells of the Tn2 phenotype, suggesting that Tn2 cells may arise when Tn0 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 Tn1 and Tn2, on the basis of the array of secreted lymphokines. Tn1 cells uniquely produce IL-2, lymphotoxin, and IFN-γ, whereas Tn2 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. Tn1 cells appear to be responsible for delayed type hypersensitivity reactions and activation of CD8+ cytolytic T lymphocytes, whereas Tn2 cells are optimal at providing help for B cell proliferation and Ab production, particularly of the IgE and IgG1 isotypes (3, 4). The Tn1 and Tn2 subsets, therefore, might mediate what has been traditionally referred to as the cellular and humoral classes of immune response, respectively.

Several lines of evidence have suggested that the mature Tn1 and Tn2 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 Tn1 and Tn2 subsets (5-7). When cloned lines are derived from a bulk population of antigen-primed lymphocytes, three predominant types of cell are obtained: Tn1 cells, Tn2 cells, and a third type, designated Tn0, 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 Tn0 clones, when maintained in culture for several weeks, eventually simplify their lymphokine profile and acquire a more typical Tn1 or Tn2 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 pluripotent Tn0 cell. This cell, under the influence of additional factors such as exogenous lymphokines and costimulatory molecules expressed by APCs, differentiates into a mature Tn1 or Tn2 effector cell.

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,1 and T,2 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,1 cells via the TCR complex are not observed in T,2 cells (13). Preliminary results have revealed that this difference may be secondary to differential expression of a particular isoform of phospholipase C (PLC, PLC-γ1 (Goldshen, R., J. Imboden, T. Gajewski, and D. Qian, unpublished observations). In addition, lymphokine production by T,1 cells is substantially more sensitive to elevations in intracellular cAMP than is lymphokine production by T,2 cells (13). The distinct signal transduction characteristics of T,1 and T,2 clones probably relate to the unique function of each of the subsets.

Stimulation of T,1 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,2 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 energizing stimuli on the functional properties and signaling characteristics of T,0 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,2-like cell. Moreover, these cells increase in size, exhibit increased basal levels of [Ca\(^{2+}\)]\(_i\), display minimal further increases in [Ca\(^{2+}\); after subsequent restimulation, consistent with a transition to a T,2-like signaling phenotype. Our observations support a model in which T,2 cells might arise when a T,0 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,2-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)propanesulfonic acid, 5 × 10\(^{-5}\) M 2-ME, 100 U/ml penicillin plus 100 μg/ml streptomycin, and additional amino acids (21). The CTLT2 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 mAbs.** 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-e) hybridoma cell line was kindly provided by Dr. J. Bluestone (University of Chicago) (23).

**T Cell Clones.** The OVA-reactive T,0 clones and the T,1 clone PGL10 were derived from DBA/2 and BALB/c mice using methods described previously (5). The conalbumin-specific T,2 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 10⁶ 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-Thyl) and M1/70 (anti-MAC-I) mAb along with low-Iox 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 μg/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 10⁶ cloned T cells in a final volume of 1 ml. For other anergy treatments, cells were stimulated with Ionomycin (1 μM) or purified B cells (6 × 10⁶) and OVA (800 μg/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 24-72 h after incubation at 37°C for 2 h. After again washing twice, each well received 10⁶ cloned T cells in a final volume of 1 ml. For other anergy treatments, cells were stimulated with Ionomycin (1 μM) or purified B cells (6 × 10⁶) and OVA (800 μg/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 24-72 h after incubation at 37°C for 2 h. After again washing twice, each well received 10⁶ cloned T cells in a final volume of 1 ml. For other anergy treatments, cells were stimulated with Ionomycin (1 μM) or purified B cells (6 × 10⁶) and OVA (800 μg/ml). Cells were collected 24–72 h later, purified by Ficoll-Hypaque centrifugation, and assayed for functional and biochemical characteristics.

**T Cell Cytolytic Assays.** Cloned cells were mixed at the indicated effector/target cell ratios with ⁴⁰Cr-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-6 concentrations were determined using a subclone of the CTLb2 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 × 10^6) purified by Ficoll-Hypaque centrifugation were stained at 4°C with FITC-coupled anti-CD4 (GK1.5), anti-CD3-e (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 FACSscan (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 μM Indo-1 acetoxymethylester for 1 h at 37°C to a final concentration of 2 × 10^5 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, 1 mM MOPS, 0.5% BSA, and 1 mM CaCl₂. Fluorescence measurements were made on samples maintained at 37°C using a spectrofluorometer (model LB-SS; 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). For A was used at a final concentration of 10 μg/ml. Cells were lysed with digitonin to determine F_m, and F_m was determined by addition of 4 mM EGTA and sufficient Tris base to raise the pH above 8.3. \[ [Ca^{2+}]_i = K_d \frac{(F - F_m)}{(F_m - F)} \], where K_d = 250 nM and F = measured fluorescence (30).

Results

Anti-CD3 mAbs, Ionomycin, or B Cells Plus Antigen Induce a Subsequent Defect in IL-2 but not IL-4 Production by T0 Clones. Unresponsiveness with respect to IL-2 production can be induced in T0 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 T2 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 energizing signal being generated in T0 but not T2 cells rather than the lack of a response of the IL-4 gene to the energizing stimulus. To address this question, we examined the effect of energizing stimuli on subsequent responsiveness of T0 clones, which produce both IL-2 and IL-4. The cell lines used were believed to be clonal based on limiting dilution frequency analysis, homogeneous morphology, and the fact that they were cloned early after derivation by micromanipulation (data not shown). Five T0 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 energized T0 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](image-url)
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.1 clones (14).

Induction of unresponsiveness to T.1 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.0 clones generated a sufficient increase in \([\text{Ca}^{2+}]\) 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.0 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.0 cells could be anergized with a more physiologic stimulus. It has been shown that anergy can be induced in T.1 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.2 but not T.1 clones in vitro (10). As depicted in Fig. 1 C for three T.0 clones, exposure to antigen and purified B cells also induced subsequent decrease in IL-4 production, exaggerating further the new lymphokine profile produced by the treated cells.

Effect of Anergizing Stimuli on Production of Additional Lymphokines by T.0 Clones. Studies of anergy with T.1 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-\(\gamma\), is marginally affected (16). Therefore, we next examined the effect of anergizing stimuli on the production of additional lymphokines by T.0 clones. For these studies, a new panel of clones was derived from DBA/2 mice, and screened for the production of IFN-\(\gamma\) in addition to IL-2 and IL-4. As shown in Fig. 2, two subtypes of T.0 clones were obtained, one which produced IFN-\(\gamma\) and the other which did not. In addition, it was noted that the frequency of IFN-\(\gamma\)-producing T.0 clones, as well as the concentration of IFN-\(\gamma\) produced by individual clones, was increased if the cells were derived in the presence of exogenous IFN-\(\gamma\) (Fig. 2). T.0 clones that produced IL-2 and IL-4 were designated T.0-A cells, and those that produced IL-2, IL-4, and IFN-\(\gamma\) were designated T.0-B cells, a nomenclature suggested previously (34). All T.0 clones examined also produced IL-3, IL-5, and TNF activity (data not shown).

Figure 2. Effect of exogenous IFN-\(\gamma\) on production of IFN-\(\gamma\) by newly derived T.0 clones. OVA-specific HTL clones were derived in the presence of syngeneic splenocytes, OVA, and either IL-2 (left) or IL-2 and IFN-\(\gamma\) (right) as described previously (5). Eight clones that produced both IL-2 and IL-4 from each group were analyzed for IFN-\(\gamma\) production by specific ELISA after stimulation with anti-CD3 mAb.

T.0-A and T.0-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.0 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-\(\gamma\) was little affected by the anergizing stimulus. Simi-
larly, IL-3 production was only modestly affected by prior exposure to anti-CD3 mAb (data not shown). The T\(_0\)-A clones that did not produce IFN-\(\gamma\) in the resting state did not acquire the ability to produce IFN-\(\gamma\) after pretreatment with anti-CD3 mAb. Similar results were obtained for several additional T\(_0\) clones of each subtype (data not shown). These results suggest that the anergizing 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\(_2\)-like phenotype could be retained after exposure of T\(_0\)-A cells to anergizing stimuli.

**T\(_0\) Cells Anergized with Anti-CD3 mAb Have Diminished Antigen-specific Cytolytic Activity.** Inasmuch as the lymphokine profile of anergized T\(_0\) cells had retained a T\(_2\)-like pattern, it was of interest to examine other functional procedures as well. The great majority of T\(_0\) and T\(_1\) clones derived in our laboratory exhibit substantial antigen-specific cytolytic activity. For T\(_0\) cells, cytolytic activity usually is greater if the cells produce IFN-\(\gamma\) as well (35, and data not shown). In contrast, many T\(_2\) 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\(_0\) clones of each subtype, when T\(_0\) 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\(_0\)-A clones, which generally lysed less well to begin with (Fig. 4, A and B).

**Surface Phenotype of Anergized T\(_0\) 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 re-stimulation of the cells was performed with immobilized anti-CD3 mAb, expression of the TCR-\(\alpha/\beta\) heterodimer, CD3-\(\epsilon\), and CD4 was examined. As shown in Fig. 5, anergized T\(_0\) cells did not express reduced levels of any of these markers, similar results were obtained with several additional T\(_0\) clones derived from BALB/c and DBA/2 mice (data not shown). These observations are in stark contrast to those obtained with T\(_1\) clones, which when anergized with anti-CD3 mAb, retained their cytolytic capability (36, and data not shown). These results suggest that exposure of T\(_0\) clones to anergizing stimuli can alter multiple functions in those cells, not only their lymphokine profile. Moreover, they suggest that stimulation of T\(_0\) cells via the TCR complex might generate a signal that does not occur after stimulation of T\(_1\) cells, one which results in disengagement of subsequent cytolytic capability.

**Effect of pretreatment with immobilized anti-CD3 mAb on the subsequent cytolytic activity of T\(_0\) cells.** Two T\(_0\)-A and T\(_0\)-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.

**Effect of treatment with immobilized anti-CD3 mAb on expression of cell surface molecules.** The T\(_0\) 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-\(\epsilon\) (b), TCR-\(\alpha/\beta\) (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 T0 cells also was examined. The Tn1 and Tn2 clones we have studied are morphologically dissimilar in that Tn2 cells are typically larger and more rounded compared to Tn1 cells, which are smaller and more club shaped. This is illustrated in Fig. 6 with photographs of a Tn1 clone PGL10 (Fig. 4 A) and the prototypic Tn2 clone D10.G4.1 (Fig. 4 B), purified by Ficoll-Hypaque centrifugation after 7 d in passage culture. The Tn0 clones resembled Tn1 cells, being relatively small and club shaped, although they typically appeared slightly larger than Tn1 cells (Fig. 4 C). Interestingly, after anergy with anti-CD3 mAb, the Tn0 cells became larger and rounder, appearing more like a Tn2 clone (Fig. 4 D). Similar results were observed after induction of anergy of Tn1 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 Tn1 and Tn2 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 Tn0 Cells. We have reported previously that, in contrast to Tn1 clones, Tn2 clones manifest little or no detectable elevation of [Ca²⁺]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 [Ca²⁺]i in Tn0 cells, which share char-

Figure 6. Morphology of Tn0 cells after induction of anergy with immobilized anti-CD3 mAb. The Tn1 clone PGL10 (A), the Tn2 clone D10 (B), the Tn0 clone 34 (C), or the Tn0 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 (10⁶), and photographed with a 35-mm camera fixed to a microscope.

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acteristics of both mature subsets. As shown in Fig. 7, after stimulation with Con A, T₁ cells responded with a 200–300% elevation in [Ca²⁺], whereas T₂ clones showed a minimal response. Interestingly, the T₀ clones exhibited an increase in [Ca²⁺], which was intermediate between that seen with T₁ and T₂ cells, ranging from 50 to 120% over resting levels. The IFN-γ-producing T₀-B clones responded with an approximately twofold greater rise in [Ca²⁺] than the T₀-A cells. The presence of a readily detectable calcium elevation in T₀ clones suggests that they employ, at least in part, a similar signal transduction apparatus to that used in T₁ cells.

For comparison, change in [Ca²⁺] 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 T₁ 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 T₂ 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 T₀ 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 T₀ cells and determine if there were alterations that correlated with the observed changes in function. This was addressed by examining changes in [Ca²⁺] in treated cells. The typical vigorous calcium flux observed with the representative T₁ clone PGL10 is depicted in Fig. 8 A, and the minimal calcium response observed with the T₀ clone D10 is represented in Fig. 8 B. Interestingly, the intermediate calcium response seen with a representative T₀-B clone (Fig. 8 C) was nearly completely abrogated after induction of anergy with anti-CD3 mAb (Fig. 8 D). Thus, anergic T₀ cells have a blunted elevation of [Ca²⁺] upon restimulation, similar to that observed with T₂ clones.

This response was examined more carefully with several additional T₀-A and T₀-B clones. When actual [Ca²⁺] concentrations were calculated, several conclusions emerged. First, the IFN-γ-producing T₀-B cells had a lower basal [Ca²⁺], compared to the T₀-A clones (Fig. 9). This result is reminiscent of observations made with T₁ and T₂ clones, the former having relatively lower [Ca²⁺] in the resting state compared to the latter (13). Second, after primary activation via the TCR complex, the [Ca²⁺] for all clones approached approximately the same plateau, around 200 nM in these experiments. Third, after anergy with anti-CD3 mAb, the basal [Ca²⁺] was markedly elevated at ~200 nM, and did not significantly increase after subsequent re-stimulation via the TCR complex (Fig. 9). This signal phenotype is similar to that observed previously with T₂ clones (13). Collectively, these results suggest that as HTL develop down the T₂ 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 T₁ 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
peptide presented either by purified class II MHC proteins.

Costimulator molecule is the essential biochemical step during TCR mAbs, the T cell mitogen Con A, and the calcium proliferation per se is the event necessary to avoid induction may be identical to the cofactor necessary for optimal proliferation of T1 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 T1 cells, anergy of T2 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 T0 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 T2 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 T2 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 T0 clones. In that study, it is argued that the cells do not acquire a T2 phenotype because the anergizing stimulus also induces reduced ability to proliferate in response to IL-4. However, T2 cells generally proliferate less vigorously in response to antigenic stimulation than T1 cells. In addition, we have observed that the majority of our clones previously characterized as T2 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 T2 clones, and is not inconsistent with the possibility that induction of anergy in T0 cells may lead to a T2-like phenotype.

Antigen-specific cytolytic activity also was inhibited in anergic T0 cells, demonstrating that more than one functional trait can be significantly affected. Interestingly, cytolytic activity was not affected after anergy of several T1 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 T1 and T0 cells may be differentially influenced by anergizing stimuli. Alternatively, T1 and T0 cells might employ distinct mechanisms of cytolyis (27), only one of which is affected in the anergic state. The fact that multiple T1-like functions were reduced after induction of anergy of T0 cells supports the notion that a T2-like phenotype is emerging under those conditions. Whether or not anergy of T0 cells renders them better able to provide help for B cell proliferation and Ab production remains to be examined.

Anergized T0 cells exhibit an elevated basal level of [Ca2+]i, and a blunted calcium flux upon restimulation, signaling attributes also possessed by T2 cells. Preliminary results have suggested that the diminished elevation of [Ca2+]i observed in T2 cells, as well as anergized T0 clones, may correlate with decreased detectable PLC-γ1 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 T0 clones also is altered, more resembling that of T2 cells (Gajewski, T., and D. Qian, unpublished observations), implying that multiple aspects of TCR-mediated signal transmission are modified in anergized T0 clones. If T2 cells indeed arise when T0 cells are exposed to anergizing stimuli, these results also suggest the possibility that the signaling characteristics of T2 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 T1-type signaling pathway. Thus, the lack of a calcium flux may correlate with the T2 phenotype, essentially being a marker for their history of being activated in the absence of a costimulator signal, but might not actually mediate the T2 phenotype.

Several APC-derived costimulator molecules and their associated receptors have been identified that may mediate costimulation and prevent induction of anergy in T1 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...
A proposed model for the ontogeny of T<sub>0</sub> and T<sub>2</sub> cells with the potential influence of anergizing stimuli is summarized in Fig. 10. Naive CD<sup>4</sup><sup>+</sup> T cells, when first activated via the TCR complex, respond with a brisk elevation in [Ca<sup>2+</sup>]<sub>i</sub> 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<sub>0</sub> phenotype. The presence of IFN-γ during this period may determine if the T<sub>0</sub> 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<sub>0</sub> cell expresses quantitatively less of the T<sub>1</sub> signaling enzymes, or that acquisition of T<sub>2</sub> traits inhibits some aspects of T<sub>1</sub> signaling. When cells at the T<sub>0</sub> stage are stimulated in the absence of a costimulator molecule, the T<sub>1</sub> program might be inhibited, giving rise to a T<sub>2</sub> phenotype. These cells then have reduced cytolytic capability and lack a substantial calcium flux. In addition, the basal level of [Ca<sup>2+</sup>]<sub>i</sub> rises as the developing cell becomes more T<sub>2</sub>-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<sub>1</sub> clones (32, 33) and inhibit the T<sub>1</sub> characteristics of T<sub>0</sub> clones (see Fig. 1). Mature T<sub>2</sub> 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<sub>1</sub> phenotype may arise when the T<sub>0</sub> cells are stimulated in the presence of a costimulator signal, perhaps under the additional influence of the cytokines IL-12 and IL-4. However, it is interesting that both naive HTL and mature T<sub>1</sub> cells exhibit a vigorous calcium flux, whereas T<sub>0</sub> cells have one of intermediate magnitude. This result suggests the possibility that T<sub>1</sub> cells may arise directly from naive CD<sup>4</sup><sup>+</sup> HTL precursors, and that the T<sub>0</sub> cell is only an intermediate in the differentiation pathway of T<sub>2</sub> 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 toleration 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<sub>0</sub> phenotype has been generated, might result not in complete anergy, but rather a T<sub>2</sub>-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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Address correspondence to Dr. Frank W. Fitch, Department of Pathology, Box 414, University of Chicago Hospitals, 5841 S. Maryland Avenue, Chicago, IL 60637.

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