Maintenance of Antigen Specificity by Human Interleukin-2-dependent T Cell Lines

Use of Antigen-presenting Cells and OKT3 Antibody in the Absence of Antigen

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Abstract

The in vitro growth of T cells obtained from localized anatomic sites of pathology may offer a new approach to the investigation of certain human autoimmune diseases. However, if interleukin-2-dependent T cell cloning is to be useful in helping to elucidate putative pathogenetic antigens in these diseases, the expansion of the small number of T cells obtainable from localized anatomic sites of pathology will often have to be accomplished in the absence of these, as yet undetermined, antigens. At present, it is a generally held belief that antigen-responsive, interleukin-2-dependent T cell lines and clones will lose antigen responsiveness if propagated in the absence of specific antigen. Thus, the use of T cell cloning might be viewed as being of limited usefulness in the investigation of certain human autoimmune diseases.

In this report we demonstrate that, when propagated in the absence of antigen, human tetanus toxoid-specific, interleukin-2-dependent T cell lines will indeed lose antigen reactivity. However, if propagated in the absence of antigen but in the presence of antigen-presenting cells, the tetanus toxoid reactivity of a subset of such lines can be maintained. Moreover, the propagation with OKT3 antibody, in addition to antigen-presenting cells, may be even more effective in maintaining antigen reactivity. These results may suggest a new approach to the use of T cell cloning technology in the investigation of certain autoimmune diseases.

Introduction

It is generally thought that antigen-specific, interleukin-2 (IL-2)[1]-dependent T cell lines and clones lose their antigen responsiveness and specificity when propagated with IL-2 in the absence of specific antigen. The kinetics and mechanisms underlying this loss, however, are unclear. The ability to maintain antigen specificity in the absence of antigen would not only be of theoretical interest but also would have practical importance in the investigation of certain human autoimmune diseases. For example, in the investigation of multiple sclerosis and rheumatoid arthritis, T cells can be propagated as IL-2-dependent lines or clones from the small number of cells available at localized anatomic sites of pathology such as cerebrospinal fluid or synovial fluid (1–4). This approach potentially could allow for the elucidation of putative localized antigen-specific T cell responses of pathogenetic significance in these diseases. However, because the antigens of pathogenetic relevance in these diseases are unknown, the generation of antigen-specific T cell lines from these localized anatomic sites by conventional means may prove theoretically difficult or impossible.

We report here that a subset of human IL-2-dependent T cell lines can in fact maintain reactivity to and specificity for tetanus toxoid (TET) when propagated in the absence of TET. The TET responsiveness and specificity of a subset of T cell lines can be maintained in the absence of TET with IL-2 and antigen-presenting cells (APC) alone. Moreover, TET specificity may be even more effectively maintained using OKT3 antibody in addition to IL-2 and APC. These results may suggest new approaches for the use of T cell cloning technology in the investigation of certain human autoimmune diseases in which only small numbers of T cells can be obtained from localized anatomic sites of pathologic involvement and in which the antigens of pathogenetic relevance are unknown.

Methods

Culture conditions. All cell cultures were performed with RPMI-1640 (Biofluids, Inc., Rockville, MD) supplemented with L-glutamine (300 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamycin (10 μg/ml) (complete media). Cultures were kept in a humidified atmosphere of 7% carbon dioxide at 37°C. Microtiter cultures were established in 96-well round-bottom plates (Costar, Cambridge, MA), and larger cultures were established in 24-well multiwell plates (Costar). Cultures were observed for growth using an inverted phase microscope.

Generation of TET-reactive T cell lines. Peripheral blood mononuclear cells were derived from tetanus toxoid immune individuals by centrifugation of heparinized whole blood over Ficoll-Hypaque. The peripheral blood mononuclear cells (1×10^6/ml) were cultured for 7 d at 37°C in RPMI-1640 with 10% fetal calf serum (individual A) or 10% human AB serum (individual B) along with TET (preservative-free tetanus toxoid—a generous gift of Dr. Frank McCarthy, Wyeth Laboratories, Inc., Marietta, PA) (2 U of flocculation L_f/ml) in a 24-well multiwell plate. After 7 d, the cells were harvested, dead cells were removed by centrifugation over Ficoll-Hypaque, and the cells were recultured (1×10^6/ml) along with fresh autologous irradiated (5,000–6,000 rad—from a ^{137}Cs source) whole peripheral blood mononuclear cells as antigen-presenting cells (2.5×10^5/ml) and TET (2 L_f/ml). After 3 d, the cells were harvested, again centrifuged over Ficoll-Hypaque, and plated in microtiter culture. These cultures, in 96-well round-bottom microtiter plates (Costar) included the twice TET-stimulated cells (80–100 cells/well) and 10^4 APC, and TET (2 L_f/ml) where appropriate. Such cultures were established with 20% IL-2 (vol/vol), and fed twice per week with fresh media and 20% IL-2, once per week or every other week where appropriate with fresh APC (10^4/well)

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1. *Abbreviations used in this paper:* AMLR, autologous mixed lymphocyte reaction; APC, antigen-presenting cells; IL-2, interleukin-2; TET, tetanus toxoid.

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and where appropriate with TET (2 L_f/ml), OKT3 antibody (10 ng/ml; Ortho Diagnostics, Raritan, NJ), or with Leu 1 antibody (10 ng/ml; Becton, Dickinson & Co., Sunnyvale, CA) every other week. When such lines demonstrated growth, they were transferred to a 24-well, multiwell plate and fed as above. In these larger wells, where appropriate, 2×10^5/well irradiated autologous whole peripheral blood mononuclear cells were added as APC.

In the investigations with individual A, the propagation of lines L3G, L3B, and L3E was carried out in the presence of 10% fetal calf serum. In the prospective investigations with individual A, one-half of the cultures was propagated in the presence of 10% fetal calf serum, and the other half propagated in the presence of 10% human AB serum. Antigen specificity was found to be maintained for longer periods with fetal calf serum. Therefore, in the investigations with individual B all lines were propagated in 10% fetal calf serum.

Interleukin-2. Our source of Interleukin-2 consisted of crude supernatants of concanavalin A-conditioned media prepared as per Northoff et al. (5). Before the supernatants that contained IL-2 were added to cultures, methyl-α-D-mannopyranoside (20 mg/ml; Calbiochem-Behring Corp., La Jolla, CA) was added to inhibit residual concanavalin A activity, and the supernatants that contained IL-2 were filtered through a filter of 0.45-μm pore size.

Proliferation assays. T cell lines were cultured in complete media with 10% human AB serum or 10% fetal calf serum—in the absence of IL-2—for 48 h before being assayed for proliferative responses. T cell lines propagated with IL-2, APC, and OKT3 antibody were always tested 2 wk after last being fed with OKT3 antibody, and T cell lines fed with biweekly APC were always tested 2 wk after last being fed with APC. T cell lines fed with biweekly APC and OKT3 were given the APC and the OKT3 antibody on the same week. [^{3}H]Thymidine incorporation proliferation assays were performed in 96-well flat-bottom microtiter plates in complete media with either 10% fetal calf serum (for lines L3G, L3B, and L3E) or with 10% human AB serum (all other T cell lines from individuals A and B). 2.5×10^4/ml T cell line cells were cultured with either 5×10^5/ml of irradiated autologous whole peripheral blood mononuclear cells (individual A—lines L3G, L3B and L3E) or the same cells after having been adhered and washed free of nonadherent cells, (individual A—all "prospective" lines) or with 10^5/ml of irradiated autologous sheep erythrocyte rosette-negative peripheral blood mononuclear cells (individual B—all T cell lines). These microtiter cultures were established with either no added exogenous antigen, or with tetanus toxoid (2 L_f/ml) or with diptheria toxoid (2–3 L_f/ml—another generous gift of Dr. Frank McCarty, Wyeth Laboratories, Inc.) or with 1% (individual A) or 2% (individual B) IL-2, (vol/vol), without other added antigen. Such cultures were kept in a humidified atmosphere of 7% CO_2 at 37°C for 3 d, and [^{3}H]thymidine (1 μCi/well; New England Nuclear, Boston, MA) was added during the last 18 h of culture. The cells were harvested with a semiautomated cell harvester, and the amount of [^{3}H]thymidine incorporated into cellular DNA measured in a beta scintillation counter (Searle-Mark III, Chicago, IL).

Sources of APC for proliferation assays. All of the APC were irradiated using 5,000–6,000 rad from a ^{137}Cs source. In the investigations involving lines L3G, L3B, and L3E from individual A, the source of APC in proliferation assays was either irradiated autologous whole peripheral blood mononuclear cells or adherent irradiated autologous whole peripheral blood mononuclear cells. The adherent cells were prepared as follows: 10^5 irradiated whole peripheral blood mononuclear cells (prepared by Ficoll-Hypaque centrifugation) in 0.1 ml of complete media with 10% fetal calf serum or 10% human serum were plated in microtiter wells that were to be used for the proliferation assays. These cells were allowed to adhere for 1–3 h at 37°C after which the microtiter wells were washed three times by gentle pipetting with Hanks' balanced salt solution. The T cell lines to be tested were then added along with antigens or IL-2 to such adherent cell monolayers.

The source of APC for the assays on the prospective studies with individual A lines was these similarly prepared adherent irradiated autologous whole peripheral blood mononuclear cells. The source of APC for the assays with lines from individual B was irradiated autologous sheep erythrocyte rosette-negative cells. Such cells were prepared as follows: peripheral blood mononuclear cells were prepared from whole blood centrifuged over Ficoll-Hypaque; such cells at 2×10^6/ml were mixed with equal volumes of a 2% (vol/vol) solution of sheep erythrocytes that had previously been treated with a 0.143 M solution of 2-aminoethylisothiouronium bromide hydrobromide. Such mixtures were incubated at 37°C for 15 min, gently pelleted by centrifugation, and allowed to sit overnight at 4°C. The next morning, these cells were centrifuged over Ficoll-Hypaque, and the cells at the interface (sheep erythrocyte rosette-negative cells) harvested, washed, and irradiated for use as APC. Such cells demonstrated a 97–99% loss of phytohemagglutinin response (before irradiation) when compared with peripheral blood mononuclear cells kept overnight at 4°C but not separated with sheep cell erythrocytes (data not shown).

Measurement of IL-2 production. T cell lines (5×10^5/ml) were co-cultured with autologous irradiated sheep erythrocyte rosette-negative cells (2×10^5/ml) in 0.2 ml of complete media with 10% human serum in 96-well flat-bottom microtiter plates (Costar). Such wells included either no added antigen, or tetanus toxoid (2 L_f/ml) or a final concentration of 1% crude IL-2-containing supernatant. These cultures were incubated at 37°C in 7% CO_2 for 22 h, at which time the supernatants were harvested and frozen at −70°C for 2–24 h. The supernatants were then thawed at 37°C and centrifuged, and 0.1 ml was plated in duplicate or triplicate round-bottom microtiter wells along with 10^4 cells/well of the IL-2-dependent mouse line—CTLL-2 (a generous gift of Dr. Kendall Smith, Dartmouth Medical School, Hanover, NH). The CTLL-2 cells were plated in complete media with 10% fetal calf serum and 2-mercaptoethanol (5×10^{-5} M). In addition, the supernatants were plated in similar wells without CTLL-2 to control for any cellular proliferation that might occur as a result of viable T cell line cells remaining in the supernatant despite freeze-thawing and centrifugation. These cultures were kept at 37°C in 7% CO_2 for 48 h and 1 μCi of [^{3}H]thymidine was added to each well 18 h before culture termination. After 48 h the cultures were harvested using a semiautomated cell harvester, and the amount of [^{3}H]thymidine incorporated into DNA was measured in a beta scintillation counter. In assessing CTLL-2 proliferation induced by such supernatants, the control wells with supernatant but without CTLL-2 never demonstrated [^{3}H]thymidine uptake at a level which effected the interpretation of the results.

Results

Overall approach. Our goals in these investigations were to define the parameters of importance in the maintenance of antigen specificity of human IL-2-dependent T cell lines. We initially generated TET-specific T cell lines from individual A (lines L3G, L3B, and L3E) that were maintained for 4 wk in the "conventional" manner (with APC and TET) and then subcultured and grown utilizing various feeding protocols. In contrast, for all subsequent investigations with lines generated from both individuals A and B, the various feeding protocols were started from the initiation of IL-2-dependent growth ("prospectively").

Our overall approach in these investigations is outlined in Table I. In studying IL-2-dependent T cell lines generated from two individuals, we used the following feeding conditions to evaluate the maintenance of antigen specificity: (*a*) the use of no APC, TET, OKT3, nor Leu 1 antibody; (*b*) the use of APC and TET; (*c*) the use of APC alone; (*d*) the use of APC and OKT3 antibody (or Leu 1 antibody as a control). All of these protocols included the use of IL-2 on a twice-weekly basis.

With the T cell lines from individual A, the APC were used only at weekly intervals and the use of Leu 1 antibody

Table I. Overall Experimental Approach

Individual A	
After 4 wk with APC and TET	No APC, TET, nor OKT3
	APC alone
	APC and TET
	APC and OKT3
Prospectively with weekly APC	No APC, TET, nor OKT3
	APC alone
	APC and TET
Individual B	
Prospectively with weekly or biweekly APC	APC alone
	APC and TET
	APC and OKT3
	APC and LEU 1

was not investigated. With the T cell lines from individual B, in addition to the feeding conditions noted above, a comparison was also made between the use of APC on a weekly schedule and the use of APC on a biweekly schedule (Table I).

Loss of TET responsiveness of TET-reactive lines. We were initially interested in investigating the kinetics of the loss of TET reactivity of lines with proven TET responsiveness. After 4 wk of growth with IL-2 and weekly stimulation with APC and TET, TET-responsive lines L3G and L3B from individual A were subcultured and subsequently fed with IL-2 but with either no TET or no APC, with just APC weekly, or with both TET and APC (both weekly). Line L3G, whose responses typify the time-related loss of TET reactivity that we commonly observed, maintained TET reactivity for the next 2 wk under all of the culture conditions (Fig. 1). However, by the 3rd wk of such growth, only the subculture of L3G that was propagated with neither TET nor APC demonstrated TET reactivity (as manifested by a stimulation index [Si—counts per minute with TET/counts per minute with media] of 4 or greater) and by the 4th wk none of the subcultures of L3G were TET responsive. (As discussed below, TET reactivity was later better maintained when TET was added biweekly rather than weekly.) As seen in Fig. 1, the lines propagated with IL-2 and APC alone, or with IL-2, APC, and TET did show transient positive responses again at the 5th wk, but then again were nonresponsive from the 6th wk on.

Most of our TET-reactive lines in these initial investigations of the kinetics of loss of TET reactivity were similarly unable to maintain TET reactivity if propagated for >2–3 wk in the absence of APC and TET. However, one line, L3B, demonstrated an unexpected response (Fig. 2). Line L3B, when propagated in the presence of IL-2 and APC alone, maintained TET reactivity for 8 wk after last being stimulated with TET. When grown in IL-2 but in the absence of both APC and TET, L3B maintained its TET reactivity for only 3 wk and remained viable for 6 wk. Line L3B grown in the "conventional fashion" with IL-2 and both APC and TET continued to maintain its TET reactivity through the 12th wk of additional culture, when it was last tested (Fig. 2).

Recently, the T3 determinant on the T cell surface has been shown to be intimately associated with the T cell antigen receptor (6, 7). We therefore were interested in determining whether antibody to the T3 determinant (OKT3 antibody), through putative indirect interaction with the T cell antigen

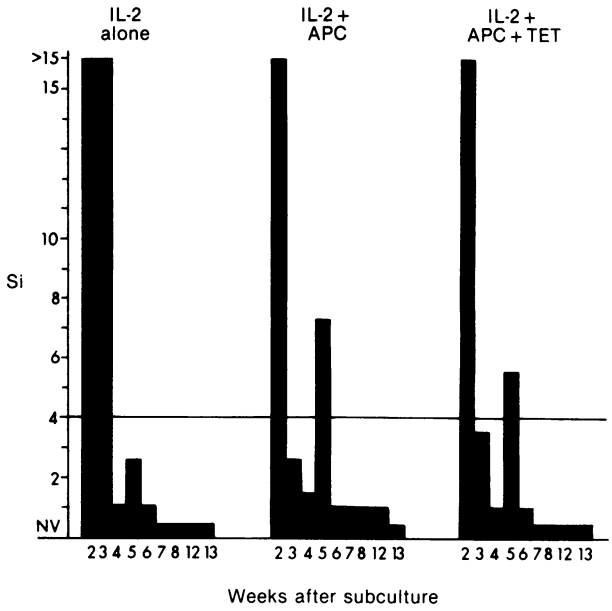

Figure 1. Sublines of line L3G. TET responsiveness. Line L3G was propagated for 4 wk with IL-2, APC, and TET, and then subcultured and propagated with either IL-2 alone, IL-2 and APC, or IL-2, APC, and TET. Sublines were assayed for TET-induced proliferation at the 2nd through 8th wk and at the 12th and 13th wk after subculture. Si, stimulation index (counts per minute with TET/counts per minute with media). An Si of 4 or greater is considered a positive response. NV, not viable.

receptor, could aid in the maintenance of antigen responsiveness of IL-2-dependent T cell lines propagated in the absence of antigen. TET responsive lines, L3B and L3E (individual A), that had been generated 4 wk earlier and grown during that time in the conventional fashion with IL-2, APC, and TET,

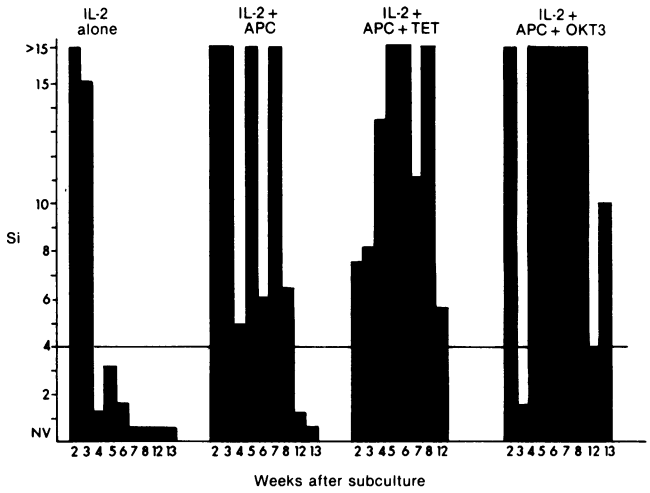

Figure 2. Sublines of line L3B. TET responsiveness. Line L3B was propagated for 4 wk with IL-2, APC, and TET, and then subcultured and propagated with either IL-2 alone, IL-2 and APC, IL-2, APC, and TET, or IL-2, APC, and OKT3 antibody. Sublines were assayed for TET-induced proliferation at the 2nd through 8th wk and at the 12th wk (and for the OKT3 antibody-propagated subline at the 13th wk) after subculture. Si, stimulation index (counts per minute with TET/counts per minute with media). An Si of 4 or greater is considered a positive response. NV, not viable.

were subcultured and subsequently propagated as above with IL-2 alone (always twice per week) or with IL-2 and APC (weekly), or in the conventional fashion with IL-2, APC (weekly), and TET (weekly). However, in addition, subcultures were propagated with IL-2, APC (weekly), and OKT3 antibody (weekly).

Line L3B, when propagated with IL-2, APC, and OKT3 antibody maintained viability and TET responsiveness through 13 wk of growth (when last tested) after last being stimulated with TET (Fig. 2). Line L3E (Fig. 3) was able to maintain TET responsiveness and viability for only an additional 2 wk of IL-2-dependent propagation after it was no longer fed with APC or TET. It maintained TET responsiveness for 3 wk after it was fed with IL-2 and APC alone and for 4 wk when propagated with IL-2, APC, and TET (again, TET had been used weekly here, rather than the optimal biweekly schedule). However, the subline of L3E propagated with IL-2, APC, and OKT3 antibody remained viable and TET-responsive through an additional 7 wk of growth (when last tested) (Fig. 3). It was noted in these proliferation assays that the background ^{3}H-incorporation (i.e., with media alone) was often very high 1 wk after the lines were fed with OKT3 antibody and back to more normal levels 2 wk after the lines were last fed with OKT3 antibody. This often led to low stimulation indices when such lines were tested 1 wk after being fed with OKT3 antibody and higher stimulation indices 2 wk after the last feed with OKT3 antibody. Therefore, we subsequently only tested lines from individual B 2 wk after last being fed with either TET or OKT3 antibody.

Prospective propagation with weekly APC alone. We next investigated the prospective frequency of TET-specific T cell lines that could maintain TET responsiveness when propagated with IL-2 and APC alone in the absence of TET. In these investigations whole peripheral blood mononuclear cells from individuals A and B were stimulated twice over a 10-d period with TET. The cells in these bulk cultures were subcultured into microtiter cultures at 80–100 cells (10-day TET-stimulated) per well, with IL-2, and with either no APC or no TET, with APC and TET, or with APC alone. Such cultures were fed twice per week with IL-2, once per week with APC, and once per week or once every other week with TET where appropriate. Thus, after the initial 10-d stimulation with TET in bulk culture, the lines that were subsequently fed with only IL-2, or with IL-2 and APC alone, were never again propagated in the presence of TET.

In those cultures fed with only IL-2 and neither APC nor TET, none of 40 microtiter wells demonstrated growth (data not shown); this mode of propagation was tested in individual A only. However in those wells fed with IL-2, APC, and TET, or with IL-2 and APC alone, all of the microtiter wells (20/20 in each group from both individuals A and B) demonstrated growth.

In evaluating the "prospective" T cell lines for TET-induced [^{3}H]thymidine incorporation, we defined a positive response as one in which all the following conditions were met: (*a*) the stimulation index (counts per minute with antigen/counts per minute with media) was 4.0 or greater; (*b*) the absolute counts per minute generated in response to antigen was at least 1,000 cpm or greater; (*c*) for lines generated from individual B, the absolute counts per minute generated in response to antigen was at least 60% of the absolute counts per minute generated in response to 2% IL-2. We found that T cell lines that met these criteria were most likely to be antigen responsive in numerous determinations over time.

With lines generated from individual A, 5 of 20 T cell lines propagated in the "conventional manner" with IL-2, APC, and TET (APC and TET given weekly) demonstrated TET reactivity when first tested at the 4th wk (Fig. 4). However, by the fifth wk all of these T cell lines had lost TET reactivity and they continued to be nonreactive over time. We were disappointed with both the frequency of lines that maintained TET reactivity and the duration of maintenance of antigen responsiveness by T cells propagated "optimally" with IL-2, APC, and TET. We subsequently found that, if we stimulated such lines with TET biweeky rather than weekly, TET-responsive T cell lines were found at a much greater frequency, and TET responsiveness was better maintained

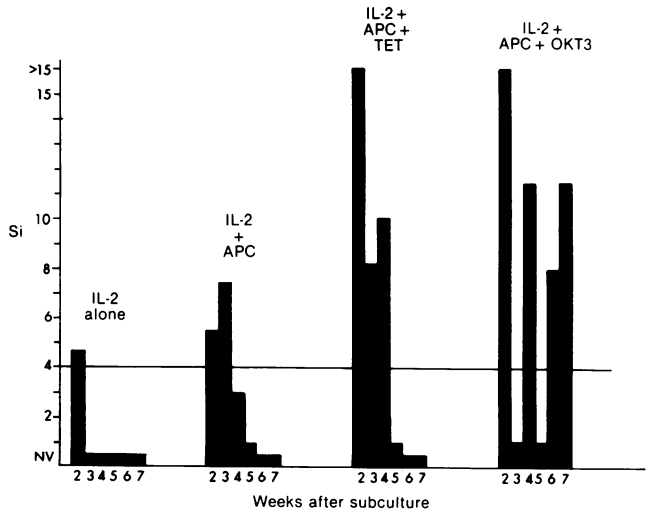

Figure 3. Sublines of line L3E. TET responsiveness. Line L3E was propagated for 4 wk with IL-2, APC, and TET, and then subcultured and propagated with either IL-2 alone, IL-2 and APC, IL-2, APC, and TET, or IL-2, APC, and OKT3 antibody. Sublines were assayed for TET-induced proliferation at the 2nd through 7th wk of subculture. Si, stimulation index (counts per minute with TET/counts per minute with media). An Si of 4 or greater is considered a positive response. NV, not viable.

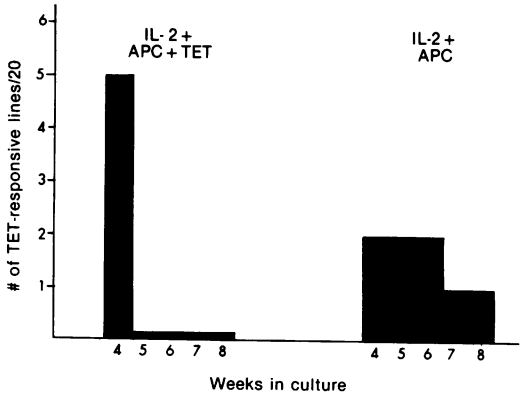

Figure 4. "Prospective" maintenance of TET responsiveness. Individual A. 10-d TET-stimulated peripheral blood mononuclear cells were grown as IL-2-dependent lines. 20 lines were propagated with IL-2, weekly APC, and weekly TET and 20 lines were propagated with IL-2 and weekly APC. Lines were assayed for TET-induced proliferation at the 4th through 8th wk of culture.

over time (see below, for individual B). Despite the poor maintenance of TET responsiveness by lines propagated with IL-2, weekly APC, and weekly TET, 2 of 20 lines from individual A that were propagated with IL-2 and APC alone proved to be TET responsive and were able to maintain TET responsiveness for up to 7 wk (line 64) and 8 wk (line 75) when last tested (Fig. 4).

By using lines generated from a second individual (individual B), we were able to confirm that a subset of T cell lines can maintain TET reactivity and specificity when propagated prospectively in the absence of TET. All of these lines were given IL-2 twice per week in the usual fashion. The frequency of T cell lines from individual B that maintained reactivity when stimulated in the conventional fashion with IL-2, TET (every other week), and APC once per week is shown in Fig. 5. By using the conventional approach (IL-2, APC, TET), at the 7th wk, 10 of 20 lines were TET responsive, and at the 9th wk, 5 of 20 lines were TET responsive. When such T cell lines were stimulated with IL-2 and APC alone (weekly), the frequency of T cell lines that maintained TET reactivity and specificity after 9 wk of growth was 4 of 20 (Fig. 5). Thus, in a second individual we were able to demonstrate that there is a definite subset of T cell lines that can maintain TET reactivity when propagated with IL-2 and APC alone in the absence of TET.

Prospective stimulation with weekly APC and biweekly OKT3 antibody. When the T cell lines of individual B were propagated with IL-2 twice per week, with APC stimulation weekly, and with biweekly stimulation with OKT3 antibody, the frequency of lines that maintained TET reactivity in the absence of TET was increased slightly over that seen with IL-2 and APC alone (Fig. 5). At the 9th wk, 6 of 20 lines demonstrated TET reactivity with IL-2, APC, and OKT3 antibody vs. 4 of 20 with IL-2 and APC alone. Thus, not only can TET reactivity be maintained in the absence of TET by propagation with IL-2 and (weekly) APC alone, but it appears that an equal or slightly greater proportion of T cell lines can maintain TET reactivity and specificity in the absence of TET when propagated with IL-2, weekly APC, and biweekly OKT3 antibody.

In contrast, T cell lines propagated with twice weekly IL-2, weekly stimulation with APC, and biweekly stimulation with Leu 1 antibody demonstrated a frequency of lines maintaining TET reactivity (2 of 20 at the 9th wk) that was less than that for lines maintained with APC alone (4 of 20) (Fig. 5). Thus, although OKT3 antibody and Leu 1 antibody both bind essentially all mature human T cells, these monoclonal antibodies were not equally effective in their ability to aid in the maintenance of TET reactivity of T cell lines propagated in the absence of TET.

Prospective propagation with biweekly APC alone. We next examined the frequency of T cell lines from individual B that could maintain TET reactivity when propagated in the absence of TET but stimulated with IL-2 (again, always given twice per week) and APC every other week. As can be seen in Fig. 6, with APC and TET both given biweekly, 14 of 20 lines were TET reactive at the 6th wk and 9 of 9 lines were TET reactive at the 10th wk. (Only nine lines were tested at the 10th wk because we chose to carry only nine of the earlier positive lines from this group.) However, by the 6th wk of growth, only 1 of 20 lines propagated with IL-2 and biweekly APC alone had maintained TET reactivity and this line remained reactive when tested at the 10th wk (Fig. 6). Thus it appears that stimulating such IL-2-propagated T cell lines with APC alone on a biweekly basis results in a lower frequency of lines maintaining TET reactivity (1 of 20 at the 6th and 10th wk—Fig. 6) than does stimulating such T cell lines with APC on a weekly schedule (4 of 20 at the 9th wk—Fig. 5).

Prospective propagation with biweekly APC and biweekly OKT3 antibody. In lines from individual B propagated with IL-2 (always twice weekly), biweekly APC, and biweekly OKT3 antibody, 5 of 20 lines at the 8th wk and 3 of 20 lines at the

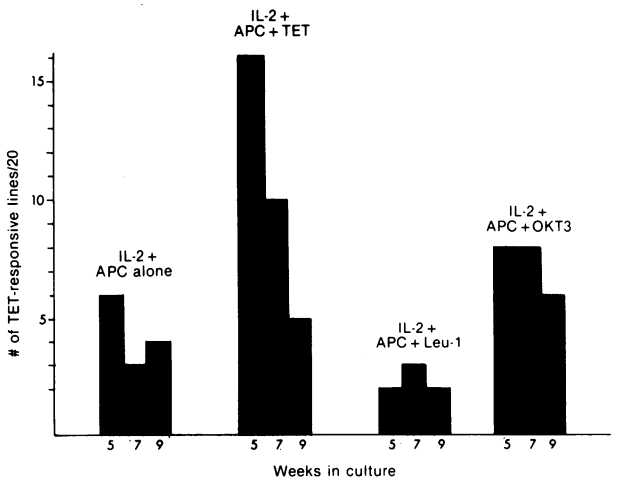

Figure 5. "Prospective" maintenance of TET responsiveness. Individual B. APC given weekly. 10-d TET-stimulated peripheral blood mononuclear cells were grown as IL-2-dependent lines. 20 lines were propagated with IL-2 and weekly APC, 20 lines were propagated with IL-2, weekly APC, and TET, 20 lines were propagated with IL-2, weekly APC, and Leu 1 antibody, and 20 lines were propagated with IL-2, weekly APC, and OKT3 antibody. Lines were assayed for TET-induced proliferation at the 5th through 9th wk of culture.

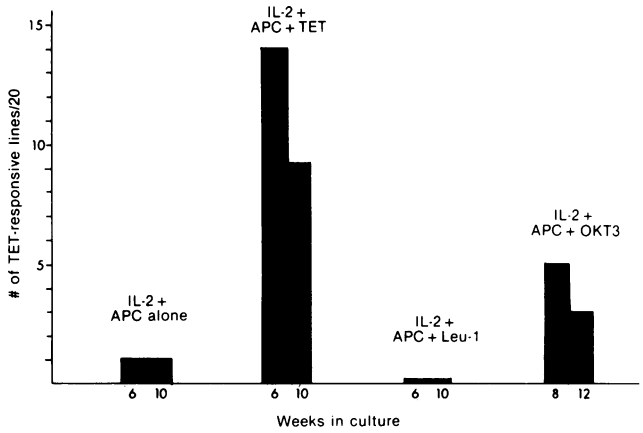

Figure 6. "Prospective" maintenance of TET responsiveness. Individual B. APC given biweekly. 10-d TET-stimulated peripheral blood mononuclear cells were grown as IL-2-dependent lines. 20 lines were propagated with IL-2 and biweekly APC, 20 lines were propagated with IL-2, biweekly APC, and TET, 20 lines were propagated with IL-2, biweekly APC, and Leu 1 antibody, and 20 lines were propagated with IL-2, biweekly APC, and OKT3 antibody. Lines were assayed for TET-induced proliferation at the 6th and 10th wk of culture, or at the 8th and 12th wk of culture.

Table II. Proliferation of T Cell Lines to Tetanus Toxoid in Association with Nonautologous APC—Individual B

Line	Propagated with	Week tested	[^{3}H]Thymidine‖			
			Autologous APC		Nonautologous APC	
			Media	TET	Media	TET
			cpm	*cpm*	*cpm*	*cpm*
29	APC*/OKT3§	8	473±413	9,408±1,236	57±6	126±79
34	APC*/OKT3§	8	60±40	9,831±746	8±3	11±4
38	APC*/OKT3§	8	43±10	3,485±152	20±9	37±14
129	APC‡	7	1,453±261	9,225±1,078	945±108	1,050±637
125	APC‡	7	1,348±436	10,941±290	845±357	693±72
77	APC‡/OKT3§	9	27±10	19,739±1,038	152±78	111±83
78	APC‡/OKT3§	9	31±14	9,362±30	12±3	17±5

* APC given biweekly. ‡ APC given weekly. § Given biweekly. ‖ Counts per minute represent the mean±standard error of duplicate or triplicate wells.

12th wk were able to maintain TET reactivity and specificity (Fig. 6). Thus, whereas the addition of OKT3 antibody to T cell lines propagated with weekly APC only slightly increased the frequency of T cell lines maintaining TET reactivity (6 of 20 vs. 4 of 20 at the 9th wk—Fig. 5), the addition of OKT3 antibody to T cell lines propagated with biweekly APC seemed to have a somewhat greater effect in increasing the frequency of T cell lines maintaining TET responsiveness in the absence of TET (5 of 20 at the 8th wk vs. 1 of 20 at the 6th wk—Fig. 6).

We also examined the frequency of T cell lines from individual B that could maintain TET reactivity when propagated in the absence of TET, but with IL-2, biweekly APC, and biweekly Leu 1 antibody. In contrast to when OKT3 antibody was used, the use of biweekly Leu 1 antibody along with biweekly APC was not more effective than biweekly APC alone in the maintenance of TET reactivity. Thus by the 6th wk none of 20 T cell lines propagated with biweekly APC and Leu 1 antibody was TET reactive (Fig. 6).

Response of T cell lines in association with non-autologous APC. As can be noted in Table II, the T cell lines of individual B that maintained TET responsiveness in the absence of TET could not be stimulated by TET in association with the nonautologous APC (irradiated sheep erythrocyte rosette-negative cells) from individual A. This suggested that these T cell lines, maintained in the absence of TET, were major histocompatability complex-restricted in their TET responses. The stimulation of individual A's T cell lines by APC from individual B was not tested.

Specificity of T cell lines. As can be noted in Tables III and IV, none of the TET-reactive lines generated from individual B that were maintained in the absence of TET responded

Table III. Maintenance of Tetanus Toxoid Reactivity by T Cell Lines Propagated in the Absence of Tetanus Toxoid—Individual B—APC Every Week

Line	Propagated with	Week tested	[^{3}H]Thymidine‡			
			Media	TET	Diphtheria	2% IL-2
			cpm	*cpm*	*cpm*	*cpm*
97	TET*	9	229±58	5,876±1,008	111±38	7,091±29
81	TET*	9	60±12	2,081±280	32±5	6,083±230
129	—	7	1,453±261	9,225±1,078	1,254±524	8,194±755
	—	9	24±10	6,041±232	8±3	4,196±86
131	—	7	52±23	138±18	45±3	9,949±209
2	Leu 1*	11	18±14	17,121±506	12±6	12,865±914
11	Leu 1*	11	23±13	78±35	9±7	7,558±538
77	OKT3*	11	40±29	11,678±251	NT	11,323±667
71	OKT3*	9	1,747±1,437	7,497±602	109±4	4,822±40
	OKT3*	11	176±105	9,736±625	NT§	6,183±542
66	OKT3*	9	58±37	262±225	332±296	5,095±340

* Given biweekly with twice weekly IL-2. ‡ Counts per minute represent the mean±standard error of duplicate or triplicate wells. § Not tested.

Table IV. Maintenance of Tetanus Toxoid Reactivity by T Cell Lines Propagated in the Absence of Tetanus Toxoid—Individual B—APC Every Other Week

Line	Propagated with	Week tested	[³H]Thymidine‡			
			Media	TET	Diphtheria	2% IL-2
			cpm	*cpm*	*cpm*	*cpm*
110	TET*	6	983±957	16,978±397	158±92	2,771±99
107	TET*	6	52±20	66±27	215±163	4,335±196
141	—	6	272±189	5,163±114	44±32	8,068±216
144	—	6	165±78	284±103	343±141	6,903±495
165	Leu 1*	6	620±152	3,689±713	729±248	8,500±1,467
161	Leu 1*	6	166±53	156±60	241±98	2,099±289
28	OKT3*	8	27±13	19±7	20±2	5,572±235
29	OKT3*	8	473±413	9,408±1,236	585±241	7,575±946
38	OKT3*	8	43±10	3,485±152	40±3	854±145

* Given biweekly with twice weekly IL-2. ‡ Counts per minute represent mean±standard error of duplicate or triplicate wells.

to diphtheria toxoid. Only one of the TET-reactive lines (line 64) from individual A was tested against diphtheria toxoid and this line similarly demonstrated no response (data not shown). This was true despite the fact that both individuals A and B demonstrated a 7-d whole peripheral blood mononuclear cell proliferative response to diphtheria toxoid (individual A: media—435±42 cpm; diphtheria—23,256±1,822 cpm; individual B: media—7,493±882 cpm; diphtheria—46,818±6,852 cpm). Thus the T cell lines carried in the absence of TET that maintained TET responsiveness also maintained TET specificity.

It was possible that irradiated T cells within the population of irradiated autologous whole peripheral blood mononuclear cells that were used as APC to feed the T cell lines might still be present in the culture over the next week, and that these APC and irradiated T cells might be producing IL-2 in response to TET at the time of assay. Such production of IL-2 seemed unlikely in that the T cell lines were always tested at least 7 d after last being fed with APC. Nevertheless, to rule out this possibility we tested a number of T cell lines for TET induced proliferation without the addition of fresh APC to the assay. None of the T cell lines tested demonstrated a response to TET in the absence of APC in the proliferation assays (data not shown).

Response of T cell lines to IL-2. It was possible that, with IL-2 and APC alone or with IL-2, APC, and OKT3 antibody, we were simply maintaining T cell lines that were extremely responsive to IL-2, and that in the proliferation assays these T cell lines were responding to TET-induced IL-2 produced by irradiated T cells remaining in the APC population. We therefore measured the proliferative response of the T cell lines to 2% exogenously added IL-2 for all T cell lines from individual B and to 1% IL-2 for some lines from individual A. We were able to demonstrate that there was no correlation of the responses to TET and the ability of any given T cell line to respond to IL-2. Thus, line 75 from individual A (Table V), when tested at the 5th wk, had responses to exogenously added IL-2 that were no greater than the IL-2 responses of many lines that were not TET responsive (such as lines 46 and 78 at the 5th wk—Table V).

Table V. "Prospective" Maintenance of Tetanus Toxoid Reactivity in the Absence of Antigen—Individual A

Line	Propagated with	Week tested	[³H]Thymidine‡		
			Media	TET	1% IL-2
			cpm	*cpm*	*cpm*
64	APC*	5	25±10	4,213±1,279	1,720±88
	APC	7	56±27	1,615±117	NT§
75	APC*	5	24±7	10,898±2,977	3,420±519
	APC	8	58±18	1,913±441	NT
6	APC*/TET*	4	216±100	22,630±1,748	NT
	APC/TET	5	88±46	5,015±442	841±10
46	APC*/TET*	5	258±76	427±50	2,876±154
78	APC*	5	40±12	34±13	4,234±428

* Given weekly with twice weekly IL-2. ‡ Counts per minute represent mean±standard error of duplicate or triplicate wells. § Not tested.

T cell lines from individual B that are representative of lines propagated with either APC (weekly) alone, APC (weekly) and OKT3 antibody, APC (weekly) and Leu 1 antibody, or with APC (weekly) and TET are shown in Table III. Lines 129 and 131 (T cell lines propagated with APC alone weekly), both had responses to 2% IL-2 at the 7th wk of 8,000–9,000 cpm, but line 129 was responsive to TET whereas line 131 was not. Similar comparisons can be made for lines 2 and 11 (propagated with APC [weekly] and Leu 1) and for lines 66 and 71 propagated with APC (weekly) and OKT3 antibody (Table III). In Table IV, which shows typical lines from individual B fed with APC every other week, similar comparisons can be made for lines 141 and 144, both propagated with APC alone, and for lines 28 and 29, both propagated with APC and OKT3 antibody. Thus it seems clear that the TET responses observed for individuals A and B represent true TET responses and not simply responses to TET-induced IL-2 generated from the APC population.

IL-2 production by TET-responsive lines. We next investigated whether the T cell lines that maintained TET reactivity and specificity during propagation in the absence of TET were producing measurable amounts of IL-2 in response to TET. We found that the majority of these lines did indeed produce measurable amounts of IL-2 in response to TET as assayed in 22-h supernatants from cultures of T cell lines, fresh irradiated autologous sheep erythrocyte rosette-negative cells as APC, and either no antigen, TET, or 1% (vol/vol) crude IL-2-containing supernatants. The latter was used both as a positive control for CTLL-2 cell responsiveness and to attempt to quantify the amount of IL-2 being adsorbed to the T cell lines. Of the T cell lines that maintained TET reactivity as determined by proliferation assays after 11 wk of propagation with weekly APC and biweekly OKT3 antibody we were able to detect IL-2 production in four of five lines tested. We have only examined IL-2 production at one concentration of T cell lines and at one time point after culture initiation, so it is still possible that the fifth T cell line was producing IL-2 in response to TET, but that in using our assay conditions this production was not detected. Similarly in lines tested after 9 wk of propagation with weekly APC alone, two of three TET-reactive (by proliferation assay) T cell lines tested were found to produce detectable amounts of IL-2 in response to TET. In lines that maintained TET reactivity when tested after 11 wk of propagation with weekly APC and biweekly Leu 1 antibody, the one TET-responsive T cell line was found to produce IL-2 in response to TET stimulation. When tested after 13 wk of growth with biweekly APC and OKT3 antibodies, four TET-responsive cell lines were tested for IL-2 production and all were found to produce IL-2 in response to TET. One of these four lines had previously proliferated in response to TET but had ceased demonstrating a proliferative response to TET after 11 wk of growth. The loss of proliferative response in this line correlated with a significant decrease in response to IL-2 (data not shown). The persistent TET-induced IL-2 production by this line, despite a loss of proliferative response to TET, was an unusual observation in our experience. Although we did not extensively test all such non-TET-proliferating, poorly IL-2-responsive T cell lines for TET-induced IL-2 production, of those few T cell lines that were examined, only the above-mentioned line demonstrated this dichotomy of responses. Examples of TET-induced IL-2 production are seen in Table VI. Of note is that irradiated autologous sheep erythrocyte rosette-negative cells used as APC were never found to produce significant amounts of IL-2 when stimulated with TET.

Discussion

It is generally believed that antigen-specific IL-2-dependent T cell lines and clones lose antigen reactivity if propagated in the absence of specific antigen. This tenet, if true, would represent a major obstacle to the use of T cell-cloning technology in the investigation of certain human autoimmune diseases in which the pathogenetically relevant antigens are unknown. In this report we have shown that, contrary to the general belief, there are subsets of antigen-responsive human T cells that can be propagated with IL-2 in the absence of specific antigen and still retain antigen responsiveness. After a 10-d stimulation of whole peripheral blood mononuclear cells with TET in bulk culture, a proportion of such T cells can be propagated as TET-specific noncloned lines by using IL-2 and

Table VI. IL-2 Production by T Cell Lines Propagated in the Absence of Tetanus Toxoid—Individual B

Line	Propagated with	Week tested	[^{3}H]Thymidine by CTLL-2‖		
			Media	TET	1% IL-2
			cpm	*cpm*	*cpm*
Erythrocyte rosette-negative, irradiated cells alone	—	11	414±90	278±95	3,490±429
129	APC*	9	131±26	7,587±622	1,996±275
2	APC*/Leu-1§	11	1,656±463	11,024±1,056	2,545±239
71	APC*/OKT3§	11	116±36	2,866±368	2,202±221
67	APC*/OKT3§	11	360±103	6,729±926	2,460±276
Erythrocyte rosette-negative, irradiated cells alone	—	13	135±64	164±59	3,168±149
29	APC‡/OKT3§	13	52±11	9,492±1,639	3,703±396
34	APC‡/OKT3§	13	84±20	6,649±956	2,571±154

* Propagated with weekly APC. ‡ Propagated with biweekly APC. § Added biweekly. ‖ Represents mean±standard error of triplicate wells of CTLL-2 cells to which 22-h supernatants from T cell lines cultured with media, TET, or 1% crude IL-2-containing supernatant were added.

APC alone or IL-2, APC, and OKT3 antibody, but in either case in the absence of TET.

The mechanism(s) by which the presence of APC alone is (are) able to maintain the antigen specificity of some of our lines has not yet been investigated. It seems unlikely that the APC are simply necessary as a nonspecific growth requirement for all long-term IL-2-dependent T cell lines in that we have generated lines both in these investigations and previously (1, 2) that grow well for long periods of time in the absence of APC. Furthermore, Barak et al. (8) have recently demonstrated that initial selection of antigen-specific human T cells on antigen-pulsed monocytes allowed for the generation of T cell lines that maintained antigen specificity for 60–80 d, despite being propagated with IL-2 only, in the absence of both antigen and APC. Our results differ from those of Barak et al. in that our lines were able to maintain antigen specificity in the absence of antigen without requiring adherence on antigen-pulsed macrophages before IL-2-dependent growth.

Individual A was last immunized with TET 10 yr before this investigation and individual B was last immunized with TET 6 yr before this investigation. It therefore seems unlikely that immunologically significant amounts of TET would routinely be found in association with the APC of either individual. Furthermore, the low levels of [^{3}H]thymidine incorporation noted in most of the "media control" proliferative responses of TET-responsive T cell lines, despite the presence of APC in these control wells, also suggests that immunologically significant amounts of TET were not routinely associated with the APC. Thus, the ability to maintain antigen reactivity of our T cell lines in the absence of antigen is not likely to be explained by the carryover of TET into the cultures by fresh APC.

One theoretical mechanism by which the presence of APC could maintain the antigen specificity of a subset of T cell lines relates to the autologous mixed lymphocyte reaction (AMLR). The AMLR is an enigmatic in vitro response of T cells, which demonstrates specificity for syngeneic major histocompatibility complex antigens (9, 10). One hypothesis invoked to explain the in vivo significance of the AMLR states that those cells responding in an AMLR also have receptors for foreign antigens. In our investigation, the maintenance of TET responsiveness of T cell lines that have been propagated with just APC in the absence of TET could be explained by such an AMLR-like mechanism. Subsets of T cells that normally require both Ia antigens (on APC) and TET for stimulation might be effectively stimulated with just Ia antigens (on APC) when propagated in the continual presence of IL-2. In this explanation such T cells would be theorized to still require both APC and TET to be stimulated in the absence of exogenous IL-2, and thus when tested in proliferation assays without IL-2, would proliferate only in response to both APC and TET and not to APC alone as in the classic AMLR.

The mechanism(s) by which the presence of OKT3 antibody may have aided in the maintenance of TET responsiveness is (are) also unclear at this time. Propagation with OKT3 may have had an effect in increasing the frequency of lines that could maintain TET specificity especially when used in conjunction with biweekly APC stimulation. With weekly APC stimulation, the effect of the addition of OKT3 was less clear. In contrast to our findings with APC and OKT3 antibody, the propagation of T cell lines with APC and Leu 1 antibody did not lead to an increase in frequency of T cell lines maintaining TET reactivity (when compared with those lines propagated with APC alone). Leu 1 antibody and OKT3 antibody both bind >95% of human peripheral blood T cells and both are monoclonal mouse IgG_{2a} antibodies. However, Leu 1 recognizes a 67,000-mol wt T cell surface determinant, whereas OKT3 recognizes a 20,000-mol wt determinant, and Leu 1 does not have the functional effects noted below for OKT3 (11). Thus, the presence of an antibody that is able to bind to the surface of T cells is itself not sufficient to increase the efficiency of maintenance of TET reactivity of T cell lines propagated in the absence of TET. The effects of OKT3 antibody noted in this report may therefore relate to the specific T3 determinant bound or to the functional effects of OKT3 antibody.

OKT3 antibody has been demonstrated to have a number of functional effects on T cells and any or all of these effects may be related to the effect described in this report. OKT3 antibody has been found to increase the IL-2 responsiveness of T cell clones (6). This effect may underlie our observations by allowing for the maintenance of a vigorous proliferative state among TET-specific cells within our lines. This in turn may prevent the overgrowth of "antigen-nonspecific" IL-2-reactive T cells within the lines, which may occur when APC and antigen are not intermittently added to the growing cells. In this regard it should be noted that although the IL-2 responsiveness of our lines that had been maintained with APC and OKT3 antibody was, on the average, slightly higher than those maintained with APC alone (data not shown), there was significant overlap in the IL-2 responses of the lines maintained under all of the conditions.

Recently, the determinant recognized by the OKT3 antibody has been reported to be intimately associated with the putative human T cell antigen receptor (6, 7). Evidence for this association includes the observed comodulation of the T3 determinant and the putative T cell antigen receptor when either were bound by their respective antibodies, and by the ability of free OKT3 antibody to inhibit antigen-specific T cell clone stimulation. It is thus possible that our findings are related to the ability of OKT3 antibody, via an interaction with this putative T3-antigen receptor complex, to maintain an "antigen-responsive" state (maintenance of expression of the antigen receptor?) when used in the context of continual IL-2 stimulation.

It should be noted that we have studied T cell lines rather than true T cell clones in these investigations. In the propagation of antigen-specific T cells from localized anatomic sites of pathology, heterogeneous IL-2-dependent T cell lines rather than true clones may be initially propagated (because the frequency of antigen-specific T cells or activated T cells in these sites is unknown). It was thus important to develop an approach that would be successful in maintaining antigen specificity in the absence of antigen even when the growing of true clones was (at least initially) unlikely. Furthermore, we believed that an approach that was successful for T cell lines would most likely be applicable to true T cell clones but that the reverse would not necessarily be the case. If the T cell lines in this report are assumed to be heterogeneous, then our results imply that a TET-specific clone or clones within our lines can maintain TET reactivity despite propagation in the absence of that antigen. Because it is a widely held belief that even true clones will lose antigen reactivity if propagated in the presence of IL-2 but in the absence of specific antigen, our results are likely to be generalizable to rigorously defined clones as well as T cell lines.

The duration of this study (~13 wk) was determined by the availability of APC. We utilized only fresh (i.e., not cryopreserved) APC for both the feeding and testing of the T cell lines. We terminated the investigation after the 13th wk because of our desire to limit further venipunctures on individuals A and B. At the end of 13 wk all of the T cell lines were, therefore, cryopreserved.

It is important to note, however, that during this 13-wk period we were able to cryopreserve large numbers of antigen-specific T cells (that had been maintained in the absence of antigen). Thus, this period of time was more than sufficient to demonstrate that our method of maintaining antigen specificity has the potential for being a practical approach. 13 wk would appear to be more than adequate time to generate large numbers of antigen-specific T cell lines from compartmentalized sites of pathology in certain autoimmune diseases.

Our results suggest that the conventionally held belief that human IL-2-dependent T cell lines cannot maintain antigen reactivity when propagated in the absence of the specific antigen may be invalid, and that subsets of T cells can be found that maintain antigen reactivity when propagated with IL-2 and APC alone or with IL-2, APC, and OKT3 antibody. The growth of human T cell lines in the absence of specific antigens but in the presence of IL-2, autologous APC, and OKT3 antibody may prove to be an important tool in the investigation of certain human autoimmune diseases. If the generally held belief that IL-2-dependent T cell lines must be propagated in the presence of the specific antigens to maintain antigen reactivity were correct, then T cell cloning from localized compartments in these diseases would most likely not prove helpful in elucidating the pathogenetically relevant antigens. In this "conventional" view the propagation of T cells from such sites under the influence of IL-2 without specific antigens would likely yield T cells without antigen responsiveness. However, the results presented here would indicate that the use of IL-2-dependent T cell cloning technology may indeed prove useful in the investigation of compartmentalized T cells in human autoimmune diseases. An approach for the use of T cell cloning in these diseases that is suggested by our results would assume that in vivo antigenic stimulation at the site of pathology is analogous to our 10-d preculture with antigen and this approach would then utilize autologous APC (weekly or biweekly) and OKT3 antibody (biweekly) in the IL-2-dependent propagation of such compartmentalized T cells. Based on our findings a proportion of the resulting T cell lines and clones could then be expected to have maintained antigen responsiveness. Such T cell lines could subsequently be used to confirm future hypotheses concerning the pathologic antigens in these diseases and perhaps also to both indirectly identify disease-related antigens and directly identify disease-related T-cell subsets via generation of monoclonal anti-T cell antigen-receptor antibodies.

The methods described in this report for maintaining antigen responsiveness of human IL-2-dependent T cell lines in the absence of antigen may therefore prove important both in our understanding of basic T cell physiology relating to the functional expression of T cell antigen receptors during IL-2-dependent T cell growth, and in helping to elucidate a number of human autoimmune diseases.

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