TRANSIENT EXPRESSION OF INTERLEUKIN 2 RECEPTORS

Consequences for T Cell Growth*

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Recent studies have revealed that T cell proliferation, although initiated by antigen or lectin, is actually mediated by a lymphocytotrophic hormone designated as interleukin 2 (IL-2)[1] (1-4). As detailed previously (5, 6), the IL-2-T cell interaction is effected by means of binding sites on membrane molecules that satisfy the criteria indicative of authentic hormone receptors, i.e., high affinity, saturability, ligand specificity, and target cell specificity. The biologic relevance of IL-2-specific receptors is reflected by the coincidence of the IL-2 binding and proliferative response curves, and further, by the finding that the rate of the T cell proliferative response is directly dependent on the concentration of IL-2 available to the cells (4, 6). Moreover, monoclonal antibodies reactive with IL-2 or the IL-2 receptor inhibit T cell proliferation by preventing IL-2-receptor binding (7, 8). The immunologic importance of the IL-2 receptor system and the unique nature of this hormone receptor mechanism stem from the finding that IL-2 receptors must be induced by appropriate immune stimuli (5, 9, 10). Thus, the immune specificity of T cell clonal expansion is guaranteed by the restriction of IL-2 receptor expression only to antigen-activated T cell clones.

The long-term culture of T cell clones depends upon an initial selection of IL-2 receptor-bearing cells, together with the provision of adequate IL-2 concentrations (3, 9-11). However, soon after the demonstration of IL-2-dependent T cell growth, it was noted that IL-2 responsiveness decayed with time (12, 13). Although this phenomenon could be prevented or reversed by the reintroduction of the original stimulating antigen, or by using lectin-containing conditioned medium, the mechanism responsible for the loss of IL-2 responsiveness was not readily apparent. Moreover, the ephemeral nature of the IL-2 responsiveness suggested that this phenomenon could be physiologically relevant to the regulation of T cell growth.

As the biologic importance of IL-2 receptors became better appreciated, it appeared that fluctuating IL-2 responsiveness might be attributable to transient cellular IL-2 receptor expression. Accordingly, with the development of the capacity to examine IL-2 receptors both quantitatively and qualitatively, this issue became amenable to analysis. Using IL-2 rendered homogeneous by mono-

clonal antibody affinity purification (7), biosynthetically radiolabeled IL-2 (5), and anti-Tac (a recently described IL-2 receptor monoclonal antibody) (8, 14, 15), the growth characteristics of activated T cells were determined and correlated with IL-2 receptor expression. IL-2 receptors appear asynchronously in lectin-activated human peripheral blood T cell populations. Moreover, upon removal of the activating lectin, IL-2 receptor levels do not persist. Rather, there occurs a time-related and IL-2-independent decay of IL-2 receptors that is rapidly reversible by restimulation with lectin. As receptor appearance and disappearance is mirrored by the proliferative rate of the cells, these observations suggest that IL-2 receptor levels are of primary importance in determining the extent of T cell clonal expansion, and consequently, the tempo and magnitude of the resultant T cell immune response.

Materials and Methods

Cell Cultures. To avoid possible variables resulting from the selection of T cell populations (introduced either as a result of the method of selection or as a result of the loss of cells during the selection process), whole peripheral blood mononuclear cell populations were chosen for study. Human peripheral blood mononuclear (PBM) cells isolated by Ficoll-Hypaque discontinuous gradient centrifugation and Jurkat subclone 6.8 cells (6) were cultured in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS) (Sterile Systems, Inc., Logan, UT), 50 U/ml penicillin G, 50 μg/ml gentamicin, 300 μg/ml L-glutamine, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selanous acid (ITS, Collaborative Research, Inc., Lexington, MA) in a humidified atmosphere of 5% CO_2 in air. PBM cells were stimulated with phytohemaglutinin (PHA) (1 μg/ml; Wellcome Reagents, Ltd., Beckenham, England) for 72 h, after which the cells were washed free of lectin and replaced into culture with receptor-saturating concentrations of IL-2 (500 pM) as indicated.

Murine IL-2-dependent cytolytic T lymphocyte lines (CTLL-2, subclone 15 G) were maintained as previously described (16) at population levels between 1×10^4 and 5×10^5 cells/ml in Iscove's modified Dulbecco's minimum essential medium (17) supplemented with 10% FCS, 50 U/ml penicillin G, 50 μg/ml gentamicin, and 500 pM human IL-2 derived from Jurkat T leukemia cells and partially purified by gel filtration (18).

Production and Preparation of Affinity-purified IL-2. Jurkat subclone 6.8 cells were routinely harvested from the exponential phase of cell growth ($0.8-1.0 \times 10^6$ cells/ml), centrifuged (250 g, 10 min), placed into serum-free DMEM (Gibco Laboratories), and cultured at 4.0×10^6 cells/ml for 14–18 h in the presence of PHA (1.5 μg/ml) and phorbol myristic acetate (50 ng/ml; Consolidated Midlands Corp., Brewster, NY). The cell-free supernatant was harvested by centrifugation (1,000 g, 15 min), filtered (0.45 μm), and stored at 4°C. Biosynthetically radiolabeled [³H]leu,lys-IL-2 was prepared as described previously (5, 6).

Radiolabeled and unlabeled IL-2 were isolated from contaminating proteins in the cell-free culture supernatants by a one-step purification protocol using an immunoaffinity column constructed with an IgG_{2a} monoclonal antibody to IL-2 (designated DMS-3) produced using the methods of de St. Groth and Scheidegger (19) as described in detail elsewhere (7). The immunoaffinity column was washed with 10 ml 0.2 N acetic acid (pH 3.0) and neutralized with 10 mM Tris-HCl, pH 7.5, before passage of Jurkat supernatants. After application of the supernatant, the column was washed with 20 column volumes each of 1 M NaCl, 10 mM Tris-HCl, pH 7.5; 0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.5; 10 mM Tris-HCl, pH 7.5; and H_2O. Bound IL-2 was eluted with 5 column volumes of 0.2 N acetic acid in two fractions (2 and 3 ml). Typically, >90% of the bound material appeared in the first fraction. The eluted protein was assayed for biologic activity and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the

presence of reducing agents according to the method of Laemmli (20) (12% acrylamide).

Radioactive proteins were visualized by fluorography using EN³HANCE (New England Nuclear, Boston, MA). Analysis of the unlabeled IL-2 eluted from the immunoaffinity adsorbant is described in detail elsewhere (7). The eluted material was comprised of a single protein (M_r, 15,500), as far as could be determined by SDS-PAGE, reverse-phase liquid chromatography, and amino-terminal amino acid sequence analysis (7).

The specific activity of the radiolabeled IL-2 was determined by relating radioactivity to biologic activity. Biologic activity and protein mass were determined on similarly purified unlabeled IL-2. Protein assays (dye binding and amino acid analysis) consistently yielded the result: 1 U biologic activity = 8.2 ng protein. Using the value of 15,500 M_r for IL-2, the specific activity of the [³H]leu,lys-IL-2 was calculated as 53,437 dpm/U or 94,400 dpm/pmol.

IL-2 Biologic Activity. IL-2 activity was determined as previously described (21) by the IL-2 concentration-dependent stimulation of proliferation of a cloned CTLL (16). CTLL proliferation, as monitored by [³H]thymidine (TdR) incorporation (2.0 μCi/ml, sp act 1.9 Ci/mM; Schwartz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY), was determined during the last 4 h of a 24-h culture period in the presence of serial twofold dilutions of a standard IL-2 preparation and the experimental sample. The dilutions that yielded 50% of the maximal CTLL [³H]TdR incorporation were determined by probit analysis; dilution values of the samples were divided by that of the standard to give the concentration of IL-2 in units per milliliter. The standard IL-2 preparation, which had been arbitrarily assigned a value of 1 U/ml, routinely yielded 50% of the maximal [³H]-TdR incorporation at a dilution of 1:10. Using a value of 8.2 ng/U, a concentration of 1 U/ml was determined to be 5.3 × 10⁻¹⁰ M.

Radiolabeled IL-2-binding Assay. Radiolabeled IL-2 binding to whole cells was performed as described in detail previously (5, 6). All cells were prepared for the assay by centrifugation, followed by incubation at 37°C in IL-2-free RPMI 1640 medium (50 ml/ 1 × 10⁷ cells) for two 1-h intervals to promote dissociation and/or degradation of endogenously bound IL-2. These conditions were chosen based upon the dissociation rate constant previously determined for intact cells and isolated plasma membranes ($t_{1/2}$ for dissociation is 25 min) (5, 6). Serial dilutions of [³H]leu,lys-IL-2 were incubated with cells (1 × 10⁶ cells/0.2 ml) in RPMI 1640 medium, 1 mg/ml bovine serum albumin (BSA) at 37°C. After a 20-min incubation, cold (4°C) RPMI 1640-BSA (1 ml) was added and the cells centrifuged (9,000 g, 15 sec). The supernatant containing the unbound fraction was removed and counted via liquid scintillation. The cell pellet was resuspended in 100 μl cold RPMI 1640-BSA and centrifuged (9,000 g, 90 sec) through a 200-μl layer of a mixture of 85% silicone oil (Dexter Hysol 550 fluid; Dexter Corp., Orlean, NY) and 16% paraffin oil (No. 0-119; Fisher Scientific Co., Fair Lawn, NJ). The tips of the tubes containing the cell pellet were cut off and counted by liquid scintillation to determine the level of bound radioactivity. The calculated values of the number of binding sites per cell were obtained by Scatchard analysis of equilibrium binding data, after subtraction of the nonsaturable binding determined in the presence of a 150-fold molar excess of unlabeled IL-2. The lower limit of detection of receptor sites per cells was 200.

Flow Microfluorometry. Expression of surface anti-Tac binding was examined by flow cytofluorometry. Cells at 10⁷/ml were incubated (1 h, 4°C) with saturating concentrations (1:5,000 dilution of ascites) of the anti-Tac monoclonal antibody (kindly provided by Dr. Takashi Uchiyama and Dr. Thomas Waldman) (8, 14, 15), washed three times, and then incubated (1 h, 4°C) with a saturating concentration (1:20 dilution) of fluorescein-conjugated rabbit anti-mouse Ig (Dakopatts; Accurate Chemical & Scientific Corp., Westbury, NY). Mature T cells were analyzed using identical methods by indirect immunofluorescence with the OKT3, OKT4, and OKT8 monoclonal antibodies (Ortho Pharmaceutical Corp., Raritan, NJ) together with fluorescein-conjugated rabbit anti-mouse Ig. For analysis of the cellular DNA content of cells, 10⁶ cells were labeled with the DNA-binding dye, propidium iodide (PI) (50 μg/ml; Calbiochem-Behring Corp., La Jolla, CA) in the presence of 100 Kunitz U/ml RNAase (Sigma Chemical Co., St. Louis, MO), 1.12%

sodium citrate, and 0.05% Nonidet P-40. Samples were passed on an Ortho cytofluoro-graph (System 50H; Ortho Diagnostic Systems, Inc., Westwood, MA) using an argon ion laser (Spectra-Physics, Inc., Mountain View, CA) at 488 nm excitation wavelength. Green fluorescence was observed using a 515–530-nm band pass filter. Red fluorescence was observed using a 630-nm-long pass filter. A minimum of 10,000 cells, gated to exclude nonviable cells, was accumulated for each histogram.

All data were analyzed using the Ortho 2150 Data Handler System (Ortho Diagnostic Systems, Inc.). For analysis of anti-Tac fluorescence, the emission was measured in relative units (channel number). A comparison of fluorescence intensity was made using the mean channel number for different histograms. The percentage of anti-Tac$^+$ cells was calculated against a background of nonspecific labeling obtained using normal Ig (1–3%). For DNA analysis, the data were plotted as linear histograms of DNA content. The relative number of cells in the G_o/G_1 phase of the cell cycle was estimated using the quick estimate method of cell cycle analysis provided with the 2150 Data Handler software.

Subsets with either high or low anti-Tac reactivity were isolated using the cell sorting facility available with the cytofluorograph. After the cell sorting procedure, unbound anti-Tac was removed from the cell suspensions by washing, and unseparated and sepa-rated cells were placed into culture in the presence of a receptor-saturating concentration of IL-2 (500 pM). [^{3}H]TdR incorporation was determined by a 2-h incubation at the initiation of the culture and after 22 h of culture in the presence of IL-2. Although anti-Tac blocks IL-2 receptor binding, this effect is antibody concentration dependent and complete suppression of the proliferative response to IL-2 requires the continuous presence of high concentrations (50 μg/ml, 3.3 × 10^{-7}M) of anti-Tac (8).

[^{3}H]TdR Incorporation.   The proportion of S phase cells in various cell populations was estimated by monitoring [^{3}H]TdR (2.0 μCi/ml; Schwartz/Mann Div., Becton, Dickinson & Co.) incorporation during 2-h culture intervals at the times indicated. Cultures were terminated by precipitation onto glass fiber filters and were counted by liquid scintillation. Data are expressed as cpm [^{3}H]TdR incorporated by 10^4 cells placed in culture.

Results

Purification of Biosynthetically Radiolabeled IL-2.   After elution and neutraliza-tion of the [^{3}H]-labeled protein bound to the DMS-3 immunoaffinity column, the fractions were tested for biologic activity. Using cloned murine, IL-2-dependent cytolytic cells and [^{3}H]TdR incorporation as an indicator of cell proliferation, serial twofold dilutions of the eluted fractions were tested. As shown in Fig. 1, the first fraction contained IL-2 activity that yielded 50% maximal CTLL [^{3}H]TdR incorporation at a sample dilution of 1:3,000, whereas the second fraction titrated in a similar fashion yielded comparable activity at a dilution of 1:435. Since the laboratory standard in this assay titered 1:22, fraction 1 contained 136 U/ml and fraction 2 contained 20 U/ml. The fractions were combined, adjusted to 20 U/ml in RPMI 1640, 1 mg/ml BSA, and examined by SDS-PAGE. As shown in Fig. 2, a fluorograph of the preparation produced a single band of radiolabeled protein at 15,500 M_r.

Time Course of Receptor Appearance and Cellular Proliferative Response to IL-2.   Previous studies have revealed that two time-related events precede the onset of T cell proliferation after lectin activation: IL-2 production and the acquisition of IL-2 receptors (9, 10, 21, 22). In these earlier studies, maximum IL-2 concentrations occurred within 24–48 h as detected by the IL-2 bioassay; however, the kinetics of IL-2 receptor appearance could only be surmised by testing for IL-2 responsiveness or absorptive capacity. Therefore, in the present study, the time course of receptor induction was examined directly, both quan-
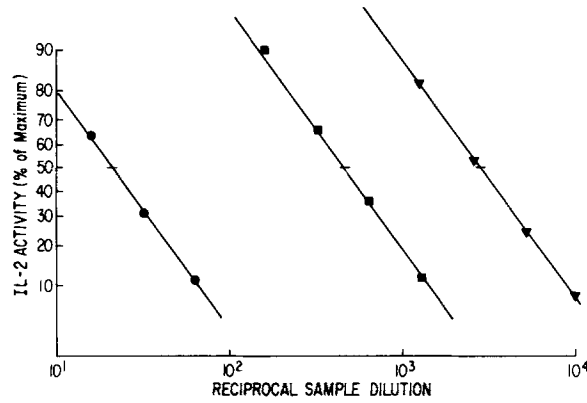
FIGURE 1. Biologic activity of immunoaffinity-purified IL-2. Murine CTLL, subclone 15H cells were cultured (4 × 10⁴ cells/ml) for 20 h with the indicated dilutions of the experimental samples (fraction 1, ▼; fraction 2, ■; laboratory standard, ●) followed by a 4-h exposure to [³H]TdR. In parallel, each sample was also tested without the [³H]TdR exposure and the difference in cpm from the two sets of cultures was used to determine cellular [³H]TdR incorporation. Maximum cpm, 23,645; minimum cpm, 22.
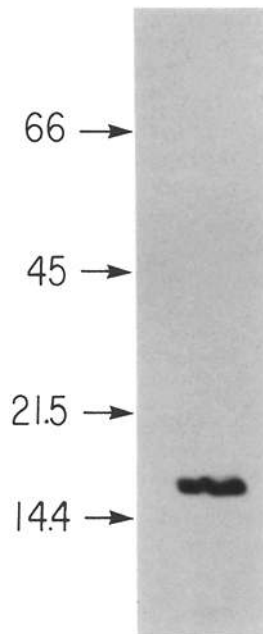

FIGURE 2. Fluorograph of SDS-PAGE analysis of immunoaffinity-purified IL-2. [³H]leu,lys-IL-2 (20,000 dpm) is shown (12% acrylamide). Molecular weight markers included lysozyme (14.4 K), soybean trypsin inhibitor (21.5 K), ovalbumin (45 K), and bovine serum albumin (66 K). Radiographic exposure time, 6 d.

titatively and qualitatively, by means of radiolabeled IL-2 binding and flow microfluorometry using anti-Tac. In addition, simultaneous measurements of [³H]TdR incorporation and cytofluorometric analysis using the DNA-binding dye, PI, provided estimates for the onset of DNA synthesis and cell cycle progression.
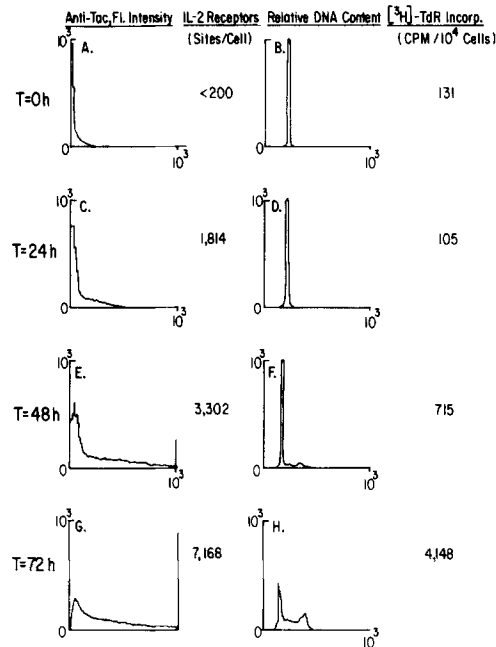
FIGURE 3. The kinetics of IL-2 receptor induction (*left*) and cell cycle progression (*right*) of PHA-stimulated human PBM. IL-2 receptor induction was examined by [³H]leu,lys-IL-2 binding and by indirect immunofluorescence using the anti-Tac monoclonal antibody and fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig. The IL-2 receptor profiles of cells cultured without lectin were indistinguishable from those of noncultured PBM. The percentage anti-Tac cells were estimated against a background of nonspecific labeling with normal mouse Ig of 1.7%. The mean channel numbers for fluorescence emissions were 37, 105, 190, and 450 for t = 0, 24, 48, and 72 h, respectively. Cell cycle distributions of human PBM at different times after exposure to PHA were monitored using the PI-fluorescence technique. [³H]TdR incorporation data were determined as described in Material and Methods.

A typical experiment, representative of five assays of the kinetics of IL-2 receptor induction and cellular proliferation after exposure of human PBM cells to PHA, is shown in Fig. 3. As reported previously, and displayed in Fig. 3A, binding of radiolabeled IL-2 and anti-Tac to freshly isolated human PBM cells was undetectable (5, 8, 14, 15). Consistent with the absence of these phenotypic markers of activation, the cells incorporated low levels of [³H]TdR and yielded a single peak of DNA staining using the PI fluorescence technique, indicative of a quiescent cell population arrested in the G_0/G_1 phase of the cell cycle (Fig. 3B). Upon the addition of lectin, detectable levels of IL-2-binding sites became evident by 6 h; by 24 h, 37% of the cells expressed anti-Tac reactivity and [³H]leu,lys-IL-2–binding sites were readily measurable (Fig. 3C). Despite the acquisition of IL-2 receptors, [³H]TdR incorporation remained at the prestimulation level and 95% of the cells remained in the G_0/G_1 phase of the cell cycle (Fig. 3D).

By the 2nd d of culture (Fig. 3E), the proportion of cells reactive with anti-Tac had increased to 47%, accompanied by a parallel increase in the mean number of IL-2 receptors detectable by the radiolabeled IL-2-binding assay. At

this time, 93% of the cells persisted in the G_o/G_1 phase of the cell cycle, which was reflected by only a modest increment in [^{3}H]TdR incorporation compared with the prestimulation level (Fig. 3F). The lack of significant proliferation during this time interval, even though detectable IL-2 receptors increased progressively, suggests that within the total population, cellular IL-2 receptor acquisition reflects a gradual and asynchronous accumulative process, and precedes the commitment to cell cycle progression.

Maximal levels of IL-2 receptors were evident after 3 d of culture (Fig. 3G). By this time, 72% of the cells were reactive with anti-Tac. Since anti-Tac reacts only with activated T cells (14, 15), and since 80% of the cells expressed phenotypic markers of mature T cells at this juncture, these data indicate that the majority (90%) of the T cells within the population expressed detectable IL-2 receptors by 72 h after stimulation with lectin. Moreover, the additional increment in the mean number of IL-2-binding sites per cell, together with the marked heterogeneity of receptor expression as shown by the anti-Tac microfluorometry profile, further support the impression that not only do the majority of cells acquire IL-2 receptors but that a progressive increase in the IL-2 receptor density per cell also takes place.

Simultaneous analysis of [^{3}H]TdR incorporation and cellular DNA content of the cell population after 72 h of culture for the first time revealed a marked increase in cycling cells. The rate of [^{3}H]TdR incorporation increased >30-fold compared with the freshly isolated cells and 51% of the cells had shifted out of G_o/G_1 (Fig. 3H).

IL-2 Dependency of Cell Cycle Progression of IL-2 Receptor$^+$ Cells. Once the majority of cells have acquired IL-2 receptors and have undergone the transition from a resting, noncycling state to an actively proliferating population in response to IL-2 produced in situ, continued cell cycle progression is dependent upon the concentration of IL-2 available. An experiment demonstrating the IL-2 concentration dependence of [^{3}H]TdR incorporation by IL-2 receptor$^+$ cells is shown in Fig. 4. In this experiment, cells harvested after 4 d of culture, expressing high levels as well as a broad range of IL-2 receptors as detected by anti-Tac staining (Fig. 4, inset), were washed and cultured in medium deprived of IL-2 for 24 h. Thereafter, the cells were exposed to affinity-purified IL-2 in concentrations ranging from 140 to 0.1 pM. After 24 h, [^{3}H]TdR incorporation, plotted as a function of the logarithm of IL-2 concentration, yielded a typical sigmoid dose-response curve. It was also notable that IL-2 receptor$^+$ cells isolated from four individuals yielded superimposable IL-2 dose-response curves.

Removal of IL-2 from an actively cycling cell population resulted in a decrease in the proportion of S phase cells within 24 h as determined by [^{3}H]TdR incorporation. If IL-2 deprivation was continued, the cells gradually accumulated in the G_o/G_1 phase of the cell cycle: after 72–96 h, the majority of the population were blocked in G_o/G_1 and resumed the morphological appearance of small quiescent lymphocytes. In contrast, cells maintained in saturating concentrations of IL-2 continued to cycle during this time period. Thus, it is evident that once lectin-activated T cells acquire IL-2 receptors, the maintenance of continued proliferation is dependent upon an adequate IL-2 concentration.

Decay of IL-2 Receptors and Cellular Proliferation. With the availability of
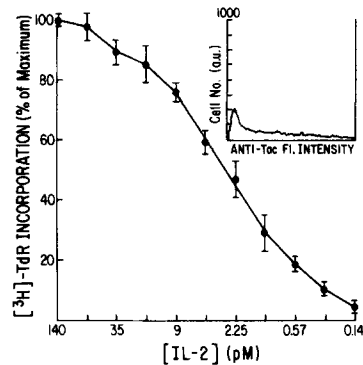
FIGURE 4. The IL-2 concentration dependence of [³H]TdR incorporation by IL-2 receptor⁺ cells. Cells harvested after 4 d of culture with PHA were washed and cultured (5 × 10⁴ cells/ ml) with the indicated concentrations of immunoaffinity-purified IL-2 for 20 h followed by a 4-h incubation with [³H]TdR. Data shown represent the mean ± SEM from cells of four individuals. Maximum cpm of [³H]TdR incorporated ranged from 5,189 to 15,306; minimum cpm (in the absence of IL-2) ranged from 251 to 4,568. Inset: anti-Tac indirect immunofluo-rescence of the cells from one of the individuals.

affinity-purified homogenous IL-2, it was possible, for the first time, to investigate the growth of lectin-activated cells once they had acquired maximal levels of IL-2 receptors. In initial experiments, after the cells had been removed from the activating signal provided by PHA and placed into long-term culture in the presence of saturating concentrations of IL-2 (500 pM, a fivefold excess of the concentration required to fully saturate IL-2 receptors [5, 6 and Fig. 4]), the rate of proliferation of the cell population slowly declined. To explore the possibility that the progressive loss of IL-2 responsiveness related to a decay in cellular IL-2 receptor levels, PHA-stimulated cells were harvested after 3 d of culture, washed, and placed at low cell density (1 × 10⁵ cells/ml) in fresh medium containing a saturating concentration of affinity-purified IL-2 (500 pM). The cells were subcultured when the cell concentration approached 1 × 10⁶ cells/ml, at which point IL-2 was replenished to ensure that the IL-2 concentration did not become growth limiting. At various time intervals after the initiation of the cultures, cell aliquots were removed and IL-2 receptor levels were determined by anti-Tac staining and the IL-2-binding assay. Simultaneously, the rate of proliferation was analyzed by PI fluorescence and [³H]TdR incorporation.

Typically, the population continued in exponential growth for several days (from the initiation of culture, 7.2 ± 0.5 d, mean ± SEM of 10 experiments) with a steady doubling time of 18–22 h. During this period, [³H]TdR incorporation rates remained relatively constant and PI analysis revealed the profile of cell populations in asynchronous exponential growth. Consistent with these growth characteristics, IL-2 receptor levels remained high as determined by both anti-Tac reactivity and [³H]leu,lys-IL-2 binding. In a representative experiment detailed in Fig. 5A, where both receptor and proliferative parameters were analyzed, the population maintained relatively constant levels of IL-2 receptors from day 3 to day 7 (7,000–9,000 sites/cell, 80–95% anti-Tac⁺ cells). Thereafter, receptor levels began to decline progressively. The loss of receptors, as deter-
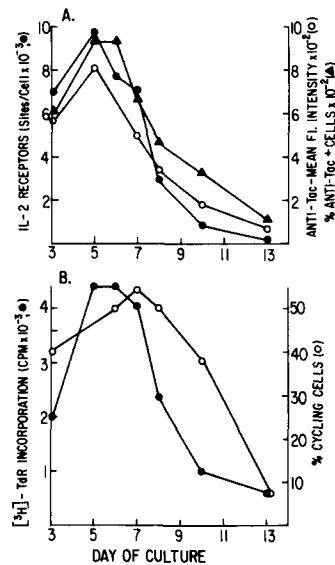
FIGURE 5. IL-2 receptor expression and cell cycle progression of IL-2 receptor⁺ cells harvested after 3 d of culture with PHA, washed, and exposed to saturating concentrations (500 pM) of affinity-purified IL-2. (A) IL-2 receptor expression as determined by [³H]leu,lys-IL-2 binding (●); anti-Tac mean fluorescence intensity (○); and the percentage of anti-Tac⁺ cells (▲). (B) Cell cycle progression as monitored by [³H]TdR incorporation (●), and cytofluorographic analysis of DNA content using propidium iodide (○).

mined by quantitative radiolabeled IL-2 binding, was paralleled by a progressive diminution of the mean fluorescence intensity of anti-Tac labeling and also by a decline in the percentage of anti-Tac⁺ cells. Typically, the decline in IL-2 receptor levels preceded a decline in the rate of cell proliferation. A fall in receptor levels was followed by a decreased rate of entry into the S phase of the cell cycle as indicated by [³H]TdR incorporation and resulted in the accumulation of the majority of the cells in G_0/G_1 by day 13 (Fig. 5B). Thus, once lectin-activated cells express IL-2 receptors, it appears that the receptor levels are maintained only transiently, after which the asynchronous population gradually loses receptors. Simultaneously, cell cycle progression declines, finally resulting in the reaccumulation of all of the cells in the population in the G_0/G_1 phase of the cell cycle.

Since the loss of IL-2 receptors occurred consistently under conditions where IL-2 concentrations were maintained at high levels, it seemed likely that IL-2-mediated receptor internalization, or the well-documented phenomenon of polypeptide hormone-mediated "down regulation" of membrane receptors (23), could account for the receptor disappearance. However, when examined directly, as displayed in Table I, IL-2 receptor levels declined progressively whether IL-2 was absent or maintained at high concentrations. These data also exclude the possibility that IL-2 bound to the receptor during the culture period masked detection of receptors by the radiolabeled IL-2-binding assay or anti-Tac fluorescence. In this respect, particular care was taken to assure the dissociation of bound IL-2: Prior to the receptor assays, cells were incubated for 2 h in the

TABLE I
IL-2-independent Loss of IL-2 Receptors*

Day of culture	[^{3}H]leu,lys-IL-2–binding sites/cell	
	IL-2 absent	IL-2 present
8	7,014	7,704
10	3,081	2,971
12	863	829

* PHA-activated human peripheral blood lymphocytes were harvested after 7 d of culture, washed, and replaced into culture in the absence and presence of exogenous, purified IL-2 (500 pM).

absence of IL-2 to allow dissociation of bound IL-2 ($t_{1/2}$ for dissociation at 37°C, 25 min) (6). Moreover, when tested directly, the data suggest that the epitope recognized by anti-Tac is not coincident with the binding site for IL-2, as IL-2 concentrations 1,000-fold in excess of those required to saturate the IL-2 receptors and 100-fold in excess of those used in the cultures (50 nM) did not inhibit anti-Tac binding even when submaximal concentrations of anti-Tac were used.

Reactivation of IL-2 Receptors and Cellular Proliferation. The transient nature of IL-2 receptor expression provided an explanation for the variable nature of IL-2 responsiveness. Given previous empirical observations that IL-2 reactivity can be restored by reintroduction of the appropriate activating signals (13), it was anticipated that the renewal of IL-2 receptor expression could explain this phenomenon. To explore this possibility, cells that no longer exhibited maximum levels of IL-2 receptors were reexposed to PHA for 2 h, washed free of lectin, and replaced into culture in the presence of purified IL-2. Measurement of IL-2 receptors after 24 h revealed that the population had regained maximum IL-2 receptor expression (Fig. 6A). Moreover, the reappearance of IL-2 receptors occurred in a more synchronous fashion after the secondary inductive signal than after the primary signal. In particular, it was notable from analyses of anti-Tac reactivity that within 24 h of second lectin exposure, the population changed from a relatively homogenous state with low levels of anti-Tac labeling (Fig. 6B) to a population with high mean [^{3}H]leu,lys-IL-2–binding levels that exhibited marked heterogeneity with respect to anti-Tac reactivity (Fig. 6C).

The reappearance of IL-2 receptors was followed by a lag phase of 24–48 h before the onset of renewed cell proliferation. This could be monitored by [^{3}H]TdR incorporation and PI analysis, both of which indicated significant cell cycle progression after the reinduction of receptors for IL-2. However, over a period of several days it was again evident that the population did not maintain IL-2 receptor expression (Fig. 6). Furthermore, this second diminution in receptor levels was accompanied by a decline in the proliferative capacity of the cells. It was possible to restimulate the cells multiple times in order to induce the appearance of IL-2 receptors (Fig. 7) and no evidence indicated that this resulted in the selection of cells that could maintain IL-2 receptor expression without lectin reactivation.

Functional Implications of Heterogeneity of IL-2 Receptor Expression. It is noteworthy that lectin-stimulated T cell populations display a marked heterogeneity of
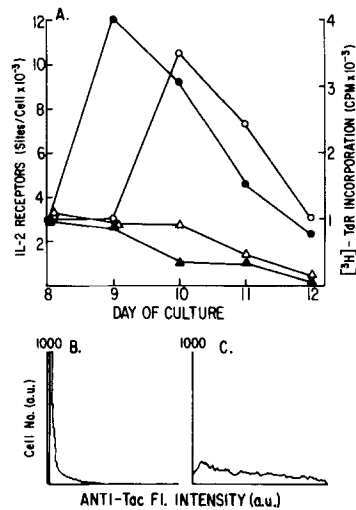
FIGURE 6. IL-2 receptor expression and proliferative response of cells after restimulation with PHA. (A) PBM cells harvested after 8 d of culture in the presence of IL-2 were restimulated with PHA (circles) or left unstimulated (triangles). Solid symbols denote [^{3}H]-leu,lys-IL-2-binding sites/cell and open symbols denote [^{3}H]TdR incorporation. (B) Histogram of anti-Tac reactivity of control cell population at day 9 of culture. (C) Histogram of PHA-stimulated cells at day 9 of culture.
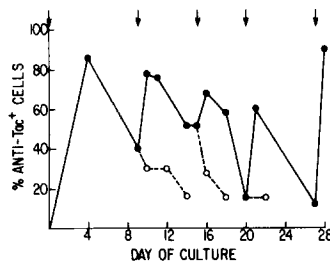

FIGURE 7. The effect of repetitive PHA stimulation on anti-Tac antigen expression by cultured human PBM cells. Arrows indicate day of exposure to PHA. Solid symbols denote restimulated cells and open symbols the unstimulated controls.

IL-2 receptor expression as determined by the anti-Tac monoclonal antibody and the cytofluorograph. This broad variation in cellular receptor density was also evident in noncycling G_0/G_1 lectin-primed T cells, suggesting that such heterogeneity is not attributable to the cell cycle asynchrony of the proliferating cell population. However, there was a correlation between the intensity of anti-Tac reactivity and the proliferative rate of cells comprising the population, suggesting that IL-2 receptor density determines the proportion of cells in the proliferative phase of the cell cycle. A typical experiment that supports this interpretation is displayed in Fig. 8 (six additional experiments yielded identical results). A cell population was synchronized into G_0/G_1 by IL-2 deprivation for 96 h, restimulated with PHA to ensure the presence of IL-2 receptors (Fig. 8A), and then separated using the cytofluorograph into subsets on the basis of low (Fig. 8B) or high (Fig. 8C) anti-Tac reactivity. The unseparated and separated
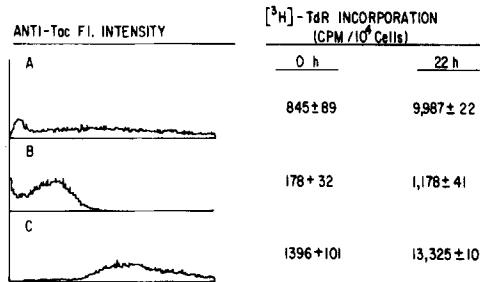
FIGURE 8.   The proliferative rate of IL-2 receptor$^+$ cell subpopulations separated on the basis of cellular IL-2 receptor density. PHA-stimulated human PBM cells were synchronized into G_0/G_1 by IL-2 deprivation for 96 h. 24 h before analysis, the cells were restimulated with PHA (0.5 μg/ml, 2 h exposure) to ensure maximum IL-2 receptor expression. The cells were then labeled with anti-Tac and fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig, and after analysis by the cytofluorograph, viable cells were selected for high or low anti-Tac reactivity. The histograms of anti-Tac reactivity are shown on the left: (A) unseparated cells, (B) low anti-Tac fluorescence intensity cells, (C) high anti-Tac fluorescence intensity cells. The [^{3}H]TdR incorporation of each population at the time of separation and after 22 h of culture with IL-2 (500 pM) is shown on the right.

cell populations were exposed to saturating concentrations of IL-2 for 22 h and monitored for proliferative response by a 2-h exposure to [^{3}H]TdR. As depicted, the rate of [^{3}H]TdR incorporation correlated with the levels of anti-Tac reactivity. Indeed, the data indicate that the high intensity anti-Tac$^+$ subset accounts for most of the proliferative activity observed in the unseparated population of cells.

Discussion

The emergence, decay, and eventual disappearance of IL-2 receptors on lectin-activated human T cells, which is followed by a parallel IL-2-dependent transient proliferative response, provides an explanation of the variability of the in vitro growth of T cells. Although the mechanism underlying the transient expression of IL-2 receptors remains obscure, IL-2-mediated down-regulation does not appear to be responsible. Rather, since repetitive exposure to the activating lectin results in continual receptor reexpression and, consequently, renewed IL-2 responsiveness, it appears that antigen/lectin recognition ultimately determines the extent of T cell clonal expansion.

For this study, radiolabeled IL-2 was purified by adsorption to the DMS-3 monoclonal anti-IL-2. Since the binding data obtained with this reagent were identical to those previously reported using biochemically purified IL-2 (5), and since both preparations manifested biologic activity with only one radiolabeled moiety detected by SDS-PAGE, the data give strong support to the conclusion that IL-2 binding and biologic activity reside in the same 15,500 M_r protein. Moreover, confirmation that the immunoaffinity-purified IL-2 is homogeneous was provided by SDS-PAGE, reverse-phase liquid chromatography, and amino acid sequence analysis, as detailed previously (7): the amino-terminal amino acid sequence of immunoaffinity-purified IL-2 coincides precisely with the sequence

predicted by cloned Jurkat (24) and normal human[2] IL-2 cDNA.

The acquisition of IL-2 receptors by the majority of T cells within 48 h, at a time when only a minor proportion of cells had entered the cell cycle, is consonant with the view that IL-2 receptor expression occurs during G_1 and precedes DNA synthesis. Moreover, the gradual increase in receptor concentration within the population that occurs before entrance into the remainder of the cell cycle suggests that a critical threshold of cellular receptor density must be attained before the commitment to DNA synthesis. These data are consistent with those of Cotner et al. (25) who recently reported that the appearance of anti-Tac reactivity preceded the onset of DNA synthesis in lectin-stimulated cultures. Furthermore, previous studies examining IL-2 receptor expression by cellular IL-2 absorption indicate that metabolic inhibition of DNA synthesis does not prevent IL-2 receptor induction (26).

Once maximal IL-2 receptor levels appear, a marked heterogeneity of IL-2 receptor expression persists within the total cell population as detected by anti-Tac binding. The anti-Tac reagent, which competitively inhibits binding of radiolabeled IL-2 to the IL-2 receptor (8), permits a qualitative assessment of IL-2 receptor expression, thus providing information unobtainable by the quantitative radioreceptor assay. Although anti-Tac has yet to be shown to be reactive solely with the IL-2 receptor, in other experiments (unpublished), similar heterogeneity in receptor profiles was observed among cloned human and murine T cell populations using IL-2 monoclonal antibodies to detect receptor-bound IL-2. Thus, the heterogeneity of receptor expression by lectin-activated human peripheral blood T cells does not necessarily reflect differences in receptor expression at the clonal level. Moreover, since receptor heterogeneity was also observed among cell populations that had accumulated in G_o/G_1, variation of receptor density with the different phases of the cell cycle does not account for this phenomenon.

While the factors that determine the rate of receptor acquisition at the level of the single cell remain obscure, the correlation of receptor levels with the proportion of cells in the actively cycling phase of the cell cycle suggests that the IL-2 receptor density per cell determines cell cycle progression, especially when IL-2 concentrations become limiting. Thus, the sigmoid shape of the IL-2 log dose-response curve could reflect the heterogeneity of receptor expression within the cell population. At low IL-2 concentrations, those cells with a high receptor density would necessarily have a greater absolute number of occupied receptors, since the proportion of receptors occupied is determined by the free IL-2 concentration. Accordingly, these data lend further support to the view that the receptor concentration per cell is a key variable in determining the commitment to DNA synthesis, and furthermore, that an accumulation of IL-2-occupied receptors to some critical threshold must occur before the onset of DNA synthesis.

The decay of IL-2 receptor levels upon removal of the cell population from the activating lectin was anticipated in view of the decline of IL-2 responsiveness

that we and others have encountered in attempts to generate IL-2-dependent long-term T cell lines (13). However, in these earlier experiments it was impossible to determine whether the loss of IL-2 responsiveness was a consequence of diminished concentrations of potential activating signals, the presence of toxic components in the IL-2-containing medium used, or an IL-2-mediated down-regulation of IL-2 receptors (23). Thus, with the availability of homogeneous, affinity-purified IL-2, it was possible to provide evidence that the loss of IL-2 responsiveness occurs as a result of an IL-2-independent, progressive decay of cellular IL-2 receptors. This conclusion is strengthened by the observation that IL-2 receptor expression is necessary for IL-2 responsiveness within a cell population, and in addition, by the observation that the IL-2-dependent proliferative response decays in parallel with IL-2 receptor loss. IL-2 receptor reexpression upon repetitive exposure to the activating lectin, followed by a renewal of IL-2-dependent proliferation, indicates decisively that the maintenance of continuous T cell clonal expansion is dependent upon the reinduction of IL-2 receptors. Furthermore, subsequent studies (unpublished) indicate similar kinetics of IL-2 receptor induction and disappearance in alloantigen-activated T cell populations. Thus, the maintenance of IL-2 receptor expression and IL-2-dependent proliferative response is strictly antigen/lectin dependent.

The kinetics of lectin-initiated IL-2 receptor appearance and disappearance observed in the present experiments are remarkably similar to those described originally by MacDonald et al. (27), who were the first to demonstrate the maintenance of alloantigen-specific murine CTL in continuous culture by repetitive alloantigen stimulation. In their study, the proliferation of alloantigen-activated CTL peaked at 4–5 d and gradually subsided to low levels by 14–21 d. Moreover, during this interval, the CTL underwent a blastogenic change in morphology followed by a gradual diminution in cell size that paralleled the decay of proliferative and cytolytic activity. Upon the reintroduction of allogeneic stimulator cells, the CTL regained maximum cytolytic activity within 24 h, before the onset of DNA synthesis. Moreover, these accelerated kinetics were also observed when in vivo primed T cells were secondarily activated in vitro. Of particular interest is the interpretation that the more rapid reinduction of cytolytic capacity after the secondary stimulation in vitro resembles an anamnestic immune response (27). In this context, "memory" T cells acquire their immune-specific cytolytic function in vitro much more rapidly than unprimed T cell populations. The rapid reappearance of maximal IL-2 receptor levels observed upon secondary stimulation is reminiscent of these early classic studies. Assuming adequate IL-2 concentrations, the more rapid acquisition of IL-2 receptors by the total cell population would explain the accelerated nature of the anamnestic response compared with the slowly progressive acquisition of IL-2 receptors demonstrated by an unprimed cell population. Consequently, it is attractive to view a memory T cell as a cell that once expressed IL-2 receptors and had subsequently lost them.

The basis for the different kinetic parameters of receptor expression between the primed and unprimed cells remains obscure. It is possible that the reactivation of primed cells results in the expression of a presynthesized receptor population whereas de novo synthesis of receptors may be required by unprimed cells.

Alternatively, it is conceivable that during the initial IL-2-dependent proliferative phase there is selection for cells that somehow express IL-2 receptors more rapidly, so that upon restimulation, receptor induction occurs synchronously. However, the salient observation remains the more rapid receptor reexpression by the primed cell population, whatever the mechanism, for this feature confers a biologic advantage to the primed cells compared with the naive population.

The immunologic relevance of the present study resides in the understanding it provides regarding the mechanism whereby external environmental stimuli direct the magnitude and extent of T cell clonal expansion by means of the IL-2 hormone receptor system. Immunologic specificity is ensured by the antigen dependence of IL-2 receptor expression. Moreover, reason dictates that IL-2 receptor expression should be only transient after each antigenic stimulation. Otherwise, with the continual exposure to new antigens, previously activated T cell clones would also be equipped to proliferate, provided adequate IL-2 concentrations are available. Thus, if the present data are extrapolated to the in vivo behavior of antigen-activated T cells, it would be anticipated that after the initial IL-2-driven expansion of a T cell clone, as antigen is cleared, IL-2 receptors within the clone would diminish, leading to the cessation of cell proliferation. Thus, it is a reasonable expectation that a further understanding of the molecular mechanism whereby antigen or lectin stimulation leads to IL-2 receptor expression, and of the factors responsible for the loss of receptors, will provide the insight essential to elucidate the regulation of both physiologic and pathophysiologic T cell immune responses.

Summary

T lymphocyte mitosis results from the interaction of interleukin 2 (IL-2) with specific receptors that appear only after appropriate immune stimulation. To assess the potential role of IL-2 receptor levels in determining the rate and magnitude of T cell proliferation, the expression of IL-2 receptors by lectin-stimulated human peripheral blood T cells was examined and correlated with T cell growth. Using biosynthetically radiolabeled IL-2 and anti-Tac, a monoclonal antibody that blocks IL-2 receptor binding, IL-2 receptors were found to accumulate slowly and asynchronously among lectin-stimulated T cells and to precede the onset of DNA synthesis. Moreover, a critical threshold of IL-2 receptor density appeared to be required before the commitment to cell cycle progression, as analyzed quantitatively by tritiated thymidine incorporation and flow cytometric analysis of cellular DNA content. Once maximal IL-2 receptor expression occurred, continued proliferation was IL-2 concentration dependent as assessed using homogenous immunoaffinity-purified IL-2. Upon removal of the activating lectin, IL-2 receptor levels progressively declined, and, in parallel, the rate of proliferation diminished. The decay of IL-2 receptors could not be attributed to IL-2-mediated down-regulation. Instead, renewed IL-2 receptor expression was dependent upon the reintroduction of the initial activating signal. Repetitive exposure to lectin resulted in a more rapid reexpression of maximal IL-2 receptor levels, which was then followed by an accelerated resumption of proliferation. Thus, the extent of T cell proliferation after immune stimulation depends upon the interplay of the IL-2 concentration available and the density

of IL-2 receptors expressed, both of which are ultimately determined by antigen/ lectin stimulation. The awareness of the transience and the antigen/lectin dependence of IL-2 receptor expression, together with the capacity to monitor T cell cultures for IL-2 receptor levels, should facilitate the initiation and maintenance of cloned, antigen-specific T cells in long-term culture. In addition, these findings suggest that, in vivo, the rapidity of acquisition of maximum IL-2 receptor levels by activated T cells and the duration of IL-2 receptor expression may well direct the magnitude of T cell clonal expansion and resultant immune responses.

References

1. Morgan, D. A., F. W. Ruscetti, and R. Gallo. 1976. Selective *in vitro* growth of T lymphocytes from normal human bone marrows. *Science (Wash. DC)*. 193:1007.
2. Ruscetti, F. W., D. A. Morgan, and R. C. Gallo. 1977. Functional and morphologic characterization of human T-cells continuously grown in vitro. *J. Immunol.* 119:131.
3. Gillis, S., and K. A. Smith. 1977. Long-term culture of tumor-specific cytotoxic T-cells. *Nature (Lond.).* 268:154.
4. Smith, K. A. 1980. T-cell growth factor. *Immunol. Rev.* 51:337.
5. Robb, R. J., A. Munck, and K. A. Smith. 1981. T cell growth factor receptors. Quantitation, specificity, and biological relevance. *J. Exp. Med.* 154:1455.
6. Smith, K. A. 1983. T-cell growth factor, a lymphocytotrophic hormone. *In* Genetics of the Immune Response. E. Moller and G. Moller, editors. Plenum Publishing Corp., New York. 151–185.
7. Smith, K. A., M. F. Favata, and S. Oroszlan. 1983. Production and characterization of monoclonal antibodies to human interleukein 2: strategy and Tactics. *J. Immunol.* 131:1808.
8. Leonard, W. J., J. M. Depper, T. Uchiyama, K. A. Smith, T. A. Waldmann, and W. C. Greene. 1982. A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor: partial characterization of the receptor. *Nature (Lond.).* 300:267.
9. Smith, K. A., S. Gillis, P. E. Baker, D. McKenzie, and F. W. Ruscetti. 1979. T-cell growth factor-mediated T cell proliferation. *Ann NY Acad. Sci.* 332:423.
10. Larsson, E. L. 1981. Mechanism of T-cell activation. II. Antigen- and lectin-dependent acquisition of responsiveness to TCGF is a non-mitogenic, active response of resting T-cells. *J. Immunol.* 126:1323.
11. Larsson, E. L., N. N. Iscove, and A. Coutinho. 1980. Two distinct factors are required for induction of T-cell growth. *Nature (Lond.).* 283:664.
12. Engers, H. D., D. Collauo, M. North, H. Von Boehmer, W. Haas, H. Hengartner, and M. Nabholz. 1980. Characterization of cloned murine cytolytic T-cell lines. *J. Immunol.* 125:1481.
13. Smith, K. A., and F. W. Ruscetti. 1981. T-cell growth factor and the culture of cloned functional T cells. *Adv. Immunol.* 31:137.
14. Uchiyama, T., S. Broder, and T. A. Waldmann. 1981. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T-cells. I. Production of

anti-Tac monoclonal antibody and distribution of Tac(+) cells. *J. Immunol.* 126:1393.

15. Uchiyama, T., D. L. Nelson, T. A. Fleisher, and T. A. Waldmann. 1981. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T-cells. II. Expression of Tac antigen on activated cytolytic T-cells, suppressor cells and one of two types of helper T-cells. *J. Immunol.* 126:1398.

16. Baker, P. E., S. Gillis, and K. A. Smith. 1979. Monoclonal cytolytic T-cell lines. *J. Exp. Med.* 149:273.

17. Iscove, N. N., and F. Melchers. 1978. Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide-reactive B lymphocytes. *J. Exp. Med.* 147:923.

18. Robb, R. J., and K. A. Smith. 1981. Heterogeneity of human T-cell growth factor (TCGF) due to variable glycosylation. *Mol. Immunol.* 18:1087.

19. de St. Groth, S. F., and D. Scheidegger. 1980. Production of monoclonal antibodies: strategy and tactics. *J. Immunol. Methods.* 35:1.

20. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.

21. Gillis, S., M. Ferm, W. Ou, and K. A. Smith. 1978. T-cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120:2027.

22. Maizel, A. L., S. R. Mehta, S. Hauft, D. Franzini, L. B. Lachman, and R. J. Ford. 1981. Human T lymphocyte/monocyte interaction in response to lectin: kinetics of entry into the S phase. *J. Immunol.* 127:1058.

23. Catt, K. J., J. P. Hardwood, G. Aquilera, and M. L. Dufau. 1979. Hormonal regulation of peptide receptors and target cell responses. *Nature (Lond.).* 280:109.

24. Taniguchi, T., H. Matsui, T. Fujita, C. Takaoka, N. Kashima, R. Yoshimoto, J. Hamuro. 1983. Structure and expression of a cloned cDNA for human interleukin 2. *Nature (Lond.).* 302:305.

25. Cotner, T., J. M. Williams, L. Christenson, H. Shapiro, T. B. Strom, and J. Strominger. 1983. Simultaneous flow cytometric analysis of human T cell activation antigen expression and DNA content. *J. Exp. Med.* 157:461.

26. Bonnard, G. D., K. Yasaka, and D. Jacobson. 1979. Ligand-activated T-cell growth factor induced proliferation: absorption of T-cell growth factor by activated T-cells. *J. Immunol.* 123:2704.

27. MacDonald, H. R., H. D. Engers, J. C. Cerottini, and K. T. Brunner. 1974. Generation of cytotoxic T lymphocytes in vitro. II. Effect of repeated exposure to alloantigens on the cytotoxic activity of long-term mixed leukocyte cultures. *J. Exp. Med.* 140:718.