INTERLEUKIN 2 REGULATES THE EXPRESSION OF TAC ANTIGEN ON PERIPHERAL BLOOD T LYMPHOCYTES

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Interleukin 2 (IL-2),[1] also known as T cell growth factor, discovered by Morgan et al. (1), is a lymphokine produced by normal peripheral blood lymphocytes after antigen or mitogen stimulation (2, 3) and is required for the proliferation and function of T cells (2, 3), natural killer cells (4–6), and cytotoxic effector cells in vitro and in vivo (7–9). In addition, IL-2 is able to induce or enhance gamma interferon production by T cells (10, 11) and granulocyte-macrophage colony-stimulating factor (Platzer, E., K. Welte, and M. A. S. Moore, manuscript in preparation). IL-2 is also produced by fresh lymphoblastic leukemic cells (12) and T cell leukemia cell lines (13).

We have demonstrated that IL-2 restores partially or completely the defective proliferative response of T cells from patients with congenital immunodeficiencies (14), acquired immunodeficiency syndrome (15), and patients after bone marrow transplantation (16).

Several laboratories including our own have purified IL-2 to homogeneity (17–19). Depending on the cellular source and stimulation conditions, IL-2 shows a molecular heterogeneity with molecular weights between 14,500 and 17,000 and isoelectric points between 6.8 and 8.2 (17, 20). The molecular heterogeneity is most likely due to variations of glycosylation of the molecule (18, 20). Recombinant DNA technology has provided recombinant IL-2 (21) with biochemical and biological characteristics similar to those of native IL-2 (22, 23).

Recently, a murine monoclonal antibody termed anti-Tac (24) was shown to be able to block the binding of IL-2 to the IL-2 receptor–positive human T cell line HUT 102 (25), and the cellular binding site for IL-2 was demonstrated to be identical with the Tac antigen, which is a glycoprotein of 58,000 mol wt (26).

OKT3 antibody recognizes 95% of peripheral blood T lymphocytes and is mitogenic in concentrations between 0.1 and 1,000 ng/ml for total T cells (27) and T cell subpopulations (28). As recently demonstrated on T cell clones (29),

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[1] *Abbreviations used in this paper:* FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; IL-2, interleukin 2; Ly-CM, lymphocyte-conditioned medium; PBMC, peripheral blood mononuclear cells; PI, propidium iodide.

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the T cell antigen receptor is a single complex consisting of the T3 molecule of 20,000–25,000 mol wt and a clonotypic, disulfide-linked heterodimer (Ti) with a molecular weight of 90,000. Anti-T3 (or OKT3 antibody) therefore triggers the proliferation of T cells in a way similar to antigen (30).

We studied the induction of Tac antigen expression with low concentrations of OKT3 antibody, which do not induce IL-2 production and no cell proliferation. Under these conditions, the expression of Tac antigen is dependent on exogenous IL-2, suggesting that IL-2 might induce its own receptor.

Material and Methods

Monoclonal Antibodies. OKT3 antibody was purchased from Ortho Pharmaceutical, Raritan, NJ. Anti-Tac antibody (ascites fluid) was kindly provided by Dr. T. Waldmann, National Insitute of Health. Monoclonal antibody against gamma interferon was prepared by one of us as described (31).

Mononuclear Cells from Peripheral Blood (PBMC). PBMC from healthy human volunteers were separated by density gradient centrifugation on Ficoll-Hypaque according to the method of Boyum (32). Cells were washed three times in RPMI 1640, supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum. Cells were kept in this medium during all subsequent incubation and washing procedures.

Adherent Cells. For adherence, up to 3×10^7 PBMC were incubated for 90 min at 37°C in 5% CO_2 in a total volume of 10 ml of Falcon tissue culture dishes (No. 3003; Falcon Labware, Oxnard, CA). Nonadherent cells were collected by gently swirling the dishes and slowly pipetting off the medium. Dishes were washed three times with medium, and adherent cells were harvested using a rubber policeman. The adherent cell fraction contained 80–95% monocytes/macrophages as judged by alphanaphthyl-acetate-esterase stain.

Separation of T Cells. For isolation of T cells, nonadherent PBMC were rosetted with neuraminidase-treated sheep erythrocytes according to a method modified from Weiner et al. (33). This fraction contained <2% monocytes/macrophages and was 90–98% positive for OKT3. This cell population is subsequently called "E^+ cells".

Treatment With Mitomycin C or Emetine. For some studies, PBMC or E^+ cells were treated with mitomycin C (Sigma Chemical Co., St. Louis, MO) or emetine-HCl (Sigma Chemical Co.) before addition to cultures. Mitomycin C is an irreversible inhibitor of DNA synthesis (34). Emetine is an irreversible inhibitor of protein synthesis at the level of translation (35). Therefore, the use of emetine was preferred over cycloheximide, since it allows the pulse exposure. Cells were incubated at concentrations of 2×10^6/ml in culture medium containing 25 μg/ml mitomycin C for 30 min at 37°C, or in 5×10^{-6} M emetine-HCl for 2 h at 37°C. After washing three times, cell concentrations were adjusted for viability as determined by trypan blue dye exclusion (viability >70% after all treatments).

Analysis of T Cell Surface Antigens (OKT3, Tac) With the Fluorescence-activated Cell Sorter (FACS). Immunofluorescence analyses were performed on a FACS IV (Becton, Dickinson & Co., Mountain View, CA). For indirect immunofluorescence, 10^6 cells were incubated with appropriate dilutions of monoclonal antibodies (1:10,000 for anti Tac; 1:40 for OKT3) for 40 min at 4°C, washed twice, and then incubated with a 1:40 dilution of affinity-purified goat anti-mouse IgG, $F(ab')_2$-fluorescein isothiocyanate (FITC) (Cappel Laboratories, Cochranville, PA) in the same way. After washing twice, cells were brought up in 1 ml phosphate-buffered saline for FACS analysis. All incubation and centrifugation steps were done at 4°C. Background fluorescence was determined using diluted normal mouse serum instead of first antibody.

Simultaneous Staining of DNA and RNA. By simultaneous staining of DNA and RNA with acridine orange the following measurements were obtained: the number of living cells, the number of dead cells, the number of cells in G_0 and G_1 phases, the number of

cells in S phase, the number of cells in G_2 plus M phases, and the mean quantity of stainable RNA per cell in the subpopulation of G_1, S and G_2 plus M. All cells were stained and measured under the same conditions (36). The measurements were performed on a modified Ortho cytofluorograf FC 201 (Ortho Diagnostic Systems, Westwood, MA).

Simultaneous Staining of DNA and Surface Antigens. Cells were prepared in the same way as for FACS analysis (see above), fixed in 70% ethanol, treated with RNase, and counterstained for DNA with propidium iodide (PI) as previously described (37). Green fluorescence (FITC-labeled secondary antibody plus Tac antibody), red fluorescence (PI-DNA), and red pulse width (nuclear diameter) were simultaneously measured on 5,000 cells per sample and analyzed using a Nova 1220 minicomputer. Red pulse width (RPW) allows us to discriminate single cells from cell aggregates; only single cells were analyzed for cell cycle distribution and Tac antigen expression. Multiparameter data analysis was done using programs developed in the Laboratory of Investigative Cytology, Sloan-Kettering Institute, New York.

Proliferation Assay. The separated E^+ cells (supplemented with 10% macrophages) or PBMC (10^6 cells/ml) were incubated in triplicate microwell cultures (No. 3596 culture plate; Costar, Cambridge, MA) in the presence or absence of mitogen (OKT3 antibody) and in the presence or absence of exogenous, highly purified IL-2. The concentrations used were: OKT3, 2.5 ng/ml (optimal concentration), 250 pg/ml, 25 pg/ml (low concentration), 2.5 pg/ml; IL-2, 20 U/ml, 200 U/ml, 2,000 U/ml. At indicated time points, 100 μl of supernatant were removed from each well to be assayed for IL-2 activity. After 72 h, identical cultures were pulsed for 4 h with [^{3}H]thymidine (0.5 μCi/well; New England Nuclear, Boston, MA) and the [^{3}H]thymidine uptake measured as described previously (28).

Assay for IL-2 Activity. For the IL-2 assay, murine IL-2-dependent cytotoxic T cells (CTLL 1) (38) were used as previously described (17). Recently, the NIH Biological Response Modifiers Program has made available an IL-2 standard. One unit of this standard is equivalent to one unit of IL-2 as previously described (17, 38).

Preparation of Purified Human IL-2. The purification of IL-2 from lymphocyte-conditioned medium (Ly-CM) has been described in detail (17). For this study we used Ly-CM purchased from Folex-Biotest-Schleussner, Inc., Fairfield, NJ. IL-2 was purified by sequential ammonium sulfate precipitation, anion exchange chromatography (DEAE cellulose, DE 52; Whatman Laboratory Products, Clifton, NJ), gel filtration on AcA 54 (LKB Instruments, Inc., Rockland, MD), and chromatography on blue agarose and on procion-red agarose (both, Bethesda Research Laboratories, Gaithersburg, MD). The final product had a specific activity of 10^6 U/mg protein and was free of detectable interferon (alpha and gamma) activities, granulocyte-macrophage colony-stimulating activity, B cell growth factor, and B cell differentiating activities. In two of six experiments detailed in Table II and two of five experiments shown in Fig. 1, recombinant IL-2, purified to homogeneity (specific activity, 2×10^6 U/mg protein) and kindly provided by AMGen, Thousand Oaks, CA, was used in parallel with highly purified IL-2. Recombinant IL-2 and highly purified IL-2 gave qualitatively and quantitatively identical results in these experiments. Therefore, results are detailed for highly purified IL-2 only.

Preparation of Human Gamma Interferon. Gamma interferon was purified from Ly-CM. The final product had a specific activity of 10^7 NIH units/mg protein.

Results

IL-2 Production and Proliferation of PBMC or E^+ Cells Plus Macrophages Stimulated with Various Concentrations of OKT3 Antibody. As shown previously (28), OKT3 antibody at concentrations of 1 ng/ml or more induces maximal IL-2 production at day 1 and maximal proliferation measured as [^{3}H]thymidine uptake in PBMC at day 3 of culture (28) (Fig. 1). As shown in Fig. 1, OKT3 antibody at a concentration of 250 pg/ml is still able to induce near optimal proliferative responses of PBMC in the absence or presence of exogenous IL-2. OKT3

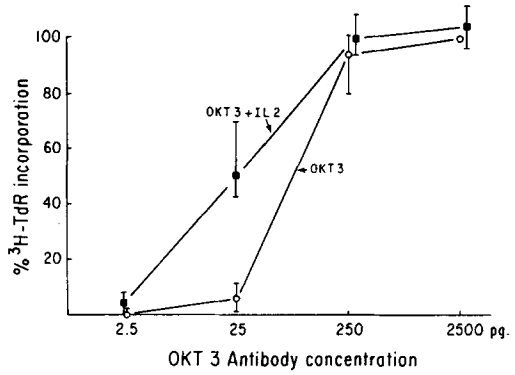

FIGURE 1. Mitogenic effect of different concentrations (2.5 pg/ml to 25 ng/ml) of OKT3 on PBMC in the presence (■) or absence (○) of IL-2 (20 U/ml). Each point is the median and range of five independent experiments. [^{3}H]Thymidine uptake was measured on day 3 of culture. For each experiment, [^{3}H]thymidine incorporation obtained with the optimal OKT3 concentration (2.5 ng/ml) in the absence of exogenous IL-2 (82,000–112,000 cpm) was considered as 100%.

antibody at a concentration of 25 pg/ml is not able to induce IL-2 production (not shown) or substantial proliferation (Fig. 1). The maximal [^{3}H]thymidine uptake at this concentration is 10% of the cpm reached with optimal OKT3 concentrations. However, in the presence of exogenous IL-2 (20 U/ml or more), this OKT3 antibody concentration leads to at least half of the maximal proliferation obtained with optimal OKT3 antibody concentrations. OKT3 antibody in concentrations of 2.5 pg/ml is not sufficient to induce a mitogenic response even in the presence of exogenous IL-2.

The proliferative response to OKT3 antibody at all concentrations tested was the same for E^+ cells supplemented with 10% macrophages as for PBMC. Optimal IL-2 production and proliferative response of E^+ cells required the presence of at least 10% macrophages. Interestingly, however, the presence of 3% macrophages was not sufficient for the induction of IL-2 production but was sufficient for E^+ cells to acquire IL-2 responsiveness when stimulated with OKT3 antibody at concentrations of 25 pg/ml or higher. IL-2 production and proliferation in response to OKT3 antibody were identical when E^+ cells with 10% macrophages or PBMC were used. Therefore, we performed all further studies with PBMC.

Cell cycle analysis was performed on PBMC after 3 d in culture in the absence or presence of IL-2. In the absence of OKT3 antibody, <3% of PBMC were in S + G_2/M phase, both with and without IL-2. When stimulated with optimal OKT3 antibody concentrations, >20% of PBMC were in S + G_2/M phase in the absence or presence of exogenous IL-2. However, PBMC stimulated with 25 pg/ml OKT3 antibody entered S phase only in the presence of exogenous IL-2 (13.2% in S + G_2/M phase vs. 1.6% in the absence of IL-2) (Table I, Fig. 3).

Time Kinetics of Proliferation Induced by Low OKT3 Antibody Concentrations. Using 25 pg/ml of OKT3 antibody did not result in a significant increase of either IL-2 production (not shown) or [^{3}H]thymidine uptake of the cells within the first 6 d in culture when compared with medium control (Fig. 2). Even at day 3, when maximal proliferation was seen with optimal OKT3 antibody concentrations, <10% of maximal proliferation occurred. IL-2 alone (in the

TABLE I

Percentage of Cells in G_0/G_1 or S + G_2/M Phase of the Cell Cycle at Day 3 of Culture

	Percentage of PBMC in:				[^{3}H]Thymidine uptake (cpm × 10^{-3})	
	G_0/G_1 phase		S + G_2/M phase			
	−IL-2	+IL-2	−IL-2	+IL-2	−IL-2	+IL-2
Medium	99.1	97.3	0.9	2.7	0.3	2
OKT3, 25 pg/ml						
Untreated cells	98.4	86.8	1.6	13.2	4	40
Mitomycin C–treated cells	98.7	98.0	1.3	2.0	0.2	0.2
OKT3, 2.5 ng/ml	79.3	74.6	20.7	25.4	93	101

Cells are unstimulated (medium) or stimulated with OKT3 antibody (25 pg/ml, 2.5 ng/ml), cultured in the absence or presence of exogenous IL-2 (20 U/ml), and analyzed by cytofluorometry after staining with acridine orange.

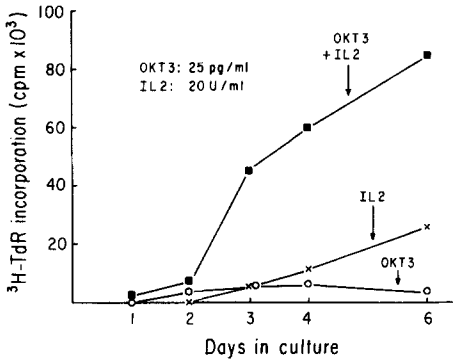

FIGURE 2. Time course of proliferation of PBMC stimulated with OKT3 antibody (25 pg/ml) in the presence (■) and absence (○) of exogenous IL-2 (20 U/ml). The proliferation of unstimulated cells in the presence of exogenous IL-2 is also shown (×).

absence of OKT3 antibody) led to an increase of proliferation starting at day 4 of culture (12,000 cpm) and to subsequently increasing cell proliferation ([^{3}H]-thymidine uptake at day 6, 25,000 cpm; Fig. 2), suggesting there were few IL-2-responsive cells in the starting cell population. OKT3 antibody (25 pg/ml) in combination with exogenous IL-2 (20 U/ml), however, led to a sharp increase of DNA synthesis and cell division already at day 3 of culture (45,000 cpm) with a subsequently increasing proliferation (Fig. 2) and long-term growth (>2 mo) of T cells by repeated addition of IL-2 to the cultures.

Since the difference between proliferation in the presence and absence of exogenous IL-2 was most pronounced at day 3 of culture, and IL-2 alone did not lead to substantial proliferation at this time point, we used cells after 3 d in culture, stimulated with 25 pg/ml OKT3 antibody, for all further studies.

Tac Antibody Immunofluorescence. The number of Tac$^+$ cells was examined on days 1–3 of culture in the presence or absence of IL-2 and containing (*a*) medium alone, (*b*) 25 pg/ml OKT3 antibody, or (*c*) 2.5 ng/ml OKT3 antibody. As shown in Table II, in the absence of IL-2 and without stimulation, <5% of the cells were Tac$^+$. IL-2 alone did not significantly increase the percentage of Tac$^+$ cells.

TABLE II
Percentage of Tac$^+$ PBMC at Day 3 of Culture

	PBMC		PBMC (mitomycin C treated)		PBMC (emetine treated)	
	−IL-2	+IL-2	−IL-2	+IL-2	−IL-2	+IL-2
Medium	<5	<6	NT	NT	NT	NT
OKT3						
25 pg/ml	6 (4–6)*	23 (18–28)*	7	21	<5	<5
2.5 ng/ml	39 (30–46)*	46 (39–49)*	38	40	<5	<5

Cells are unstimulated (medium) or stimulated with OKT3 antibody (25 pg/ml, 2.5 ng/ml). The viability of PBMC at the initiation of and at day 3 of culture was >70% in all experiments and regardless of treatment with mitomycin C or emetine. NT, not tested.

* Results from six independent experiments are shown as median and range.

TABLE III
Time Kinetic of Tac Antigen Expression

	OKT3 (25 pg/ml)		OKT3 (2.5 ng/ml)	
	−IL-2	+IL-2	−IL-2	+IL-2
Day 1	4.6	6.0	14.1	18.8
Day 2	4.5	12.4	24.4	26.7
Day 3	6.7	21.5	30.1	39.5

PBMC were cultured in the presence of OKT3 antibody (25 pg/ml, 2.5 ng/ml), and in the presence or absence of IL-2 (20 U/ml). Results are expressed as percent Tac$^+$ PBMC as determined by FACS analysis.

Using OKT3 antibody in a concentration of 25 pg/ml, the percentage of Tac$^+$ cells was still 6% or less in the absence of IL-2 at days 1–3 in culture (Table III); however, upon stimulation with exogenous IL-2, 6% of PBMC were Tac$^+$ at day 1, 12.4% at day 2, and 21.5% at day 3. In five additional experiments using OKT3 antibody at a concentration of 25 pg/ml, between 4 and 6% of PBMC were Tac$^+$ at day 3 of culture in the absence of exogenous IL-2, and between 18 and 28% of PBMC were Tac$^+$ in the presence of exogenous IL-2. Stimulation with optimal OKT3 concentrations (2.5 ng/ml) led to between 14 and 19% Tac$^+$ cells in the absence or presence of exogenous IL-2, respectively, at day 1 of culture, 24 and 27%, respectively, at day 2 of culture, and 30 and 40%, respectively, at day 3 of culture (Table III). In five additional experiments using optimal OKT3 antibody concentrations, the number of Tac$^+$ cells were 39% (range, 30–46%) or 49% (range, 39–56%) in the absence or presence of IL-2, respectively. Control experiments showed that OKT3 antibody at all concentrations used in this study did not result in detectable background immunofluorescence.

Simultaneous Staining of DNA and Tac Antigen. As shown in Fig. 3, the independently achieved results of proliferative response and the percentage of Tac$^+$ cells were confirmed by measuring simultaneously DNA fluorescence and immunofluorescence with Tac antibody. In the upper panel, results are shown

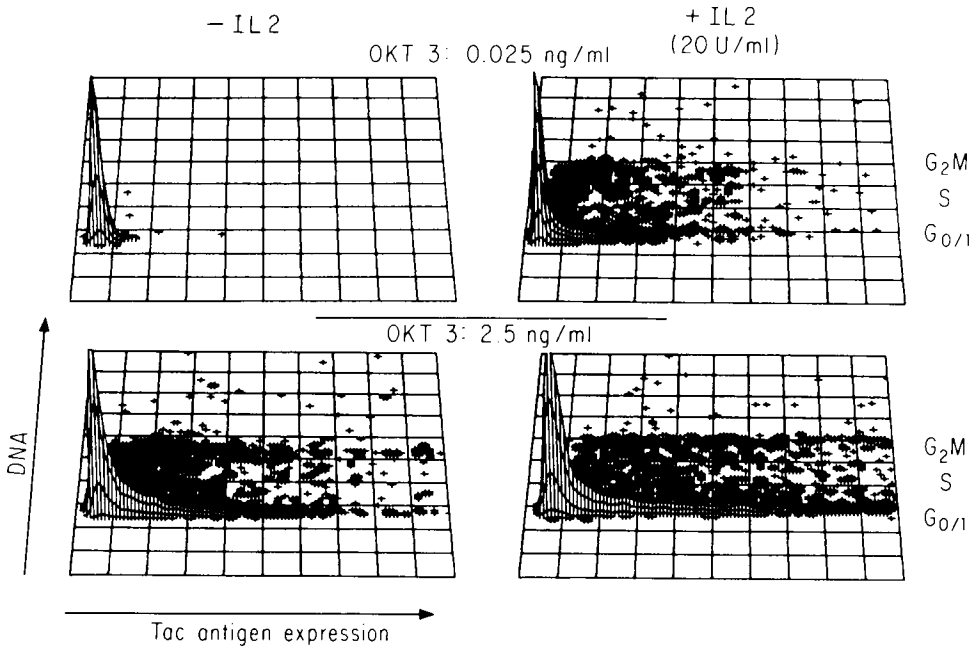

FIGURE 3. Simultaneous measurement of cellular DNA fluorescence (propidium iodide–stained DNA) and immunofluorescence obtained by staining with Tac antibody plus $F(ab')_2$-FITC goat anti-mouse IgG. (*Top*) Pattern when 25 pg/ml OKT3 antibody was used; (*bottom*) pattern when 2.5 ng/ml OKT3 antibody was used. (*Left*) Pattern in the absence of exogenous IL-2; (*right*) pattern in the presence of exogenous IL-2 (20 U/ml).

for stimulation with low OKT3 antibody concentrations (25 pg/ml). The left side of the upper panel demonstrates that the cells in the absence of exogenous IL-2 do not enter S phase and do not express Tac antigen. In the presence of exogenous IL-2 (right side, upper panel), ~13% of PBMC entered S + G_2/M phase. All of them showed immunofluorescence with Tac antibody. Using optimal OKT3 antibody concentrations, as shown in the lower panel of Fig. 3, a significant proportion of cells expressed Tac and proliferated, as detailed above. Even at this concentration of OKT3 antibody, however, the density of Tac antigen was higher in the presence of IL-2 than in its absence.

Effect of DNA Synthesis and Protein Synthesis Inhibitors on the Appearance of Tac$^+$ Cells in OKT3-stimulated Cultures. As shown in Table II, treatment with mitomycin C, a DNA synthesis inhibitor, for 30 min at 37°C did not change the appearance of Tac$^+$ cells in the OKT3-stimulated cell population compared with untreated cells. The majority of cells, however, did not enter the S or G_2M phase of the cell cycle (Table II). The expression of Tac antigen can be attributed to exogenous IL-2 since (*a*) the concentration of OKT3 antibody used (25 pg/ml) did not lead to endogenous IL-2 production and (*b*) there is no or very low Tac antigen expression in the absence of exogenous IL-2 (Fig. 3).

Treatment of the cells with emetine, an irreversible inhibitor of protein synthesis, for 2 h at 37°C resulted in the total prevention of Tac antigen expression at all concentrations of OKT3 antibody used in our study, suggesting the requirement for protein synthesis for Tac antigen expression.

TABLE IV
Effect of Exogenous IL-2 on Tac Antigen Expression and [^{3}H]thymidine Uptake

IL2	Tac$^+$ cells	[^{3}H]thymidine uptake (cpm × 10^{-3})
U/ml	%	
0	<5	7
20	23	69
200	28	71
2,000	18	62

PBMC were stimulated with OKT3 antibody (25 pg/ml) in the presence or absence of varying concentrations of IL-2. Percentage of Tac$^+$ cells was determined by FACS analysis.

Effect of Different Concentrations of IL-2 on Tac Antigen Expression. As shown in Table IV, the number of Tac$^+$ cells in PBMC stimulated with 25 pg/ml OKT3 antibody and IL-2 at concentrations of 20, 200, and 2,000 U/ml were 23, 28, and 18%, respectively. The corresponding [^{3}H]thymidine uptakes were 69,000, 71,000, and 62,000 cpm, respectively. In the absence of IL-2, <5% of the cells were Tac$^+$ and the [^{3}H]thymidine uptake was 7,100 cpm.

Role of Gamma Interferon in the Responsiveness to IL-2 and Tac Antigen Expression. Since some evidence has been reported (39) that gamma interferon plays a role in IL-2 receptor expression, we studied the effect of highly purified gamma interferon and monoclonal antibody against gamma interferon on T cell responsiveness to IL-2 and Tac antigen expression. Neither gamma interferon in concentrations of 20, 40, and 100 U/ml nor anti-gamma interferon (100 neutralizing units/ml) significantly altered the proliferative response to exogenous IL-2 or the percentage of Tac$^+$ cells under our experimental conditions (data not shown).

Discussion

In this study we describe the effect of low, nonmitogenic concentrations of OKT3 antibody in combination with optimal concentrations of exogenous IL-2 on the proliferation of PBMC or E$^+$ cells (supplemented with 10% autologous macrophages). We have chosen this combination because (*a*) OKT3 antibody alone at this concentration does not induce measurable endogenous IL-2 production, allowing us to study the effect of exogenous IL-2, (*b*) it does not induce IL-2 receptor expression as measured by Tac antibody FITC immunofluorescence analysis, and (*c*) exogenous IL-2 in combination with this low OKT3 antibody concentration leads to proliferation of PBMC or E$^+$ cells in the presence of macrophages. Therefore, we consider this a good model to study the effect of exogenous IL-2 on Tac expression without interference by endogenous IL-2 (the possibility that IL-2 induces its own production is under investigation). IL-2 alone was not able to induce proliferation and Tac antigen expression within the first 3 d of cultures (Fig. 2).

We used OKT3 as stimulus because OKT3 antigen is associated with or is part of the T cell antigen recognition complex (29). Therefore, stimulation with

OKT3 antibody triggers IL-2 production and proliferation of T cells in a way analogous to antigenic stimulation. In a concentration of 25 pg/ml, OKT3 antibody is not able to induce proliferation or Tac expression (Figs. 1, 3) but in combination with exogenous IL-2 there is Tac expression and a proliferative response. However, only about half of the maximal proliferation obtained with optimal OKT3 concentrations is seen under these conditions, as measured by both [^{3}H]thymidine uptake and the percentage of cells in S + G_2/M phase by flow cytometry.

The observation that OKT3 antibody does not induce IL-2 production at 25 pg/ml is consistent with a recent report (30) that for IL-2 production and release by a cloned T cell line, T cell antigen receptor crosslinking is required, whereas for IL-2 receptor expression, T cell antigen receptor crosslinking is not required. OKT3 at concentrations of 25 pg/ml, or ~10 molecules of IgG per cell under our experimental conditions, is not likely to be able to crosslink the antigen receptor and, therefore, IL-2 production will not occur.

We studied the effects of exogenous IL-2 in combination with low OKT3 antibody concentrations on the proliferation of PBMC over a period of 6 d in cultures (Fig. 2). We chose day 3 for most of our more detailed studies, since optimal OKT3 concentrations induce maximal proliferation at this time point under our experimental conditions (28) and exogenous IL-2 alone in the absence of mitogens is still without effect on proliferation on day 3.

Whether this difference between proliferation in the presence and absence of IL-2 might be used as a simple IL-2 assay to determine the amount of IL-2 in mitogen-free unknown samples is under study. Preliminary results demonstrate that as low as 0.5 U/ml IL-2 can be detected with this assay system, which is comparable in sensitivity to the murine cytotoxic T lymphocyte line (CTLL) assay (38 and data not shown).

The difference in responsiveness of PBMC at day 3 of culture to low OKT3 antibody concentrations (25 pg/ml) in the presence and absence of IL-2 is consistent with the different number of Tac^+ cells detected under these conditions. In the absence of IL-2, the same number of cells were Tac^+ in the medium control and in low OKT3 antibody concentrations (6% or less). However, in the presence of IL-2, the control medium also showed only 6% Tac^+ cells, whereas with IL-2 in combination with low OKT3 antibody concentrations, 18 and 28% of the cells were Tac^+.

To address the question whether Tac^+ T cells are derived from a small number of Tac^+ cells present at the initiation of cultures and grown up in the IL-2-containing medium, we treated T cells with mitomycin C. Treatment with mitomycin C, a potent inhibitor of DNA synthesis, still allowed for expression of Tac antigen to the same extent as without treatment (Table II), but prevented the cells from entering DNA synthesis and cell division (Table I). This suggests that IL-2 in combination with low OKT3 antibody concentration does not simply lead to proliferation of preexisting Tac^+ cells, resulting in a higher number of Tac^+ cells at day 3. Rather, it induces the expression of Tac antigen on previously Tac^- cells that need not necessarily enter DNA synthesis.

Further evidence that Tac^+ cells do not simply derive from Tac^+ cells present at the initiation of culture is that, after stimulation with optimal OKT3 antibody

concentrations, a high percentage of Tac^+ cells were already present at day 1 of culture, when [^{3}H]thymidine uptake was still low or not detectable and cells had not entered the S phase of the cell cycle. Since after stimulation with optimal OKT3 concentrations, maximal IL-2 production occurs at day 1 of culture (28), the Tac antigen expression at this time might also be due to endogenous IL-2. Similar results were reported by Uchiyama et al. (24), where concanavalin A was used as mitogen. It was also reported by the same group (24) that treatment of the cells with anti-Tac antibody plus complement before initiation of culture did not abolish the appearance of Tac^+ cells when stimulated with a mitogen or in a cytotoxicity assay.

The receptor induction seems dependent on protein synthesis, since pretreatment with emetine, an irreversible inhibitor of protein synthesis at the level of translation, prevented any proliferative response to IL-2 and expression of Tac antigen, without influencing the percentage of viable cells significantly.

In summary, the results obtained in our study strongly suggest that IL-2 is involved in the induction of its own receptor. Tac antigen expression appears to be a two-step event: preactivation by OKT3 of cells capable of activating Tac antigen, which sensitizes these cells to the Tac-inducing effects of IL-2. IL-2 is then required for the full expression of functionally active Tac antigen, leading to cell proliferation. Among the possible mechanisms explaining this phenomenon are the following: (*a*) OKT3 antibody in low concentration (25 pg/ml) triggers an activation of the IL-2 receptor not detectable by the Tac antibody immunofluorescence technique. As a second step, the presence of exogenous IL-2 results in the expression of the Tac antigen, now detectable with Tac antibody. (*b*) Binding of OKT3 antibody, even at this low concentration, may lead to an alteration of the cell membrane polarity by influencing the ion influx or efflux (e.g., Ca^{++}) and the presence of IL-2 in the medium then triggers activation of Tac antigens in the altered membrane. (*c*) Another membrane antigen may react with IL-2 that is distinct from the Tac antigen and that may also be required for IL-2-driven proliferative responses. This would be consistent with a report (40) about a T cell antigen reacting with IL-2 on guinea pig T cells that is different from the IL-2 receptor. This complex distinct from the IL-2 receptor might play a critical role in the initial processing of the IL-2 signal. IL-2 might activate this distinct antigen which, in concert with the activated T3 complex or antigen recognition complex, then leads to the Tac antigen expression.

The observation that low concentrations of OKT3 antibody, even in the presence of optimal IL-2 concentrations (up to 2,000 U/ml), will only lead to half-maximal Tac expression and proliferation suggests that only a subpopulation of PBMC is able to respond under these conditions, while other cells require higher concentrations of OKT3 antibody to acquire IL-2 responsiveness.

We cannot exclude the possibility that IL-2 regulates the expression of its own receptor via another lymphokine or monokine, such as receptor-inducing factor or gamma interferon. Johnson and Farrar (39) reported that gamma interferon is able to induce the expression of IL-2 receptors on human peripheral blood T cells. We therefore studied the effect of highly purified gamma interferon in concentrations between 20 and 100 U/ml and anti–gamma interferon monoclonal antibody (100 neutralizing units) in the presence and absence of IL-2 and

low OKT3 antibody concentrations (25 pg/ml). There was no significant alteration of proliferation, measured by [^{3}H]thymidine uptake at day 3 of culture or by the percentage of Tac$^+$ cells at the same time point, suggesting no major role for gamma interferon in Tac induction under our experimental conditions. These results are at variance with the findings of Johnson and Farrar (39), possibly due to differences in experimental conditions.

Results similar to the effects of low concentrations of OKT3 antibody combined with exogenous IL-2 on normal PBMC were obtained with optimal concentrations of OKT3 on PBMC from patients with leukemia and aplastic anemia after bone marrow transplantation. The proliferative response of PBMC to OKT3 antibody (2.5 ng/ml) from 23 patients was 8,000 cpm (median) in the absence of exogenous IL-2 and 51,000 cpm (median) in the presence of exogenous IL-2 (16). This suggests a functional defect of the transplanted lymphocytes or their precursors in these patients. The near-normal response in the presence of exogenous IL-2, however, would be consistent with the fact that IL-2 is able to induce its own receptor even on these functional defective cells, leading to proliferation of the cells.

It would be of great interest to know whether IL-2 can regulate its own receptor expression also in vivo. We have initiated studies to investigate the percentage of Tac$^+$ total PBMC and T cell subsets from patients with immunodeficiency states who are enrolled in our ongoing phase 1 trial of IL-2 (41).

Summary

We investigated the effect of OKT3 antibody and interleukin 2 (IL-2) on Tac antigen expression and the proliferation of human peripheral blood mononuclear leukocytes. OKT3 monoclonal antibody at low, nonmitogenic concentrations (25 pg/ml) or IL-2 alone at optimal concentrations (20 U/ml) did not induce IL-2 receptor expression, as measured by Tac antibody or by T cell proliferation. However, costimulation with these concentrations of OKT3 antibody and IL-2 led to Tac antigen expression and T cell proliferation. These data suggest that the T cells are activated in two steps: OKT3 antibody at 25 pg/ml does not induce Tac antigen expression, but preactivates T cells to become responsive to IL-2. The addition of exogenous IL-2 then leads to expression of the IL-2 receptor, as recognized by Tac antibody, and to subsequent proliferation.

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