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INDUCTION OF IL 2 RECEPTOR EXPRESSION AND CYTOTOXICITY OF THYMOCYTES BY STIMULATION WITH TCF1[1]

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We investigated the role of T cell cytotoxicity inducing factor 1 (TCF1) in the induction of a cytotoxic T cell response. We found that help-deficient thymocyte cultures supplied with saturating amounts of purified IL 2 did not develop CTL in a 5-day culture. The expression of cytotoxicity was dependent on the addition of TCF1 derived from the T cell hybridoma K15. TCF1 also induced proliferation of thymocytes in the presence of IL 2. Only the PNA$^-$ thymocyte subpopulation responded to TCF1 with proliferation and cytotoxicity in the presence of IL 2. The monokine IL 1 also induced proliferation in this subpopulation but failed to induce cytotoxicity. IL 1 was further distinguished from TCF1 by inhibition of IL 1-induced but not TCF1-induced proliferation by anti-IL 1 antibodies. In addition, using anti-IL 2 receptor antibodies (AMT 13), we showed that TCF1 in the presence of IL 2 substantially increased IL 2 receptor expression in thymocytes. IL 1 had the same effect on induction of IL 2 receptor expression as TCF1. Because some effects of IL 1 and TCF1 are distinct and some overlap, we discuss whether IL 1 and TCF1 induce different subsets of PNA$^-$ thymocytes.

The development of mature cytotoxic T lymphocytes (CTL)[2] from immature CTL precursors (CTLp) is a complicated process that involves the activity of several cell types and soluble mediators. Ia$^+$ macrophages process antigen and secrete interleukin 1 (IL 1). IL 1 is essential for the activation of T helper cells (1). Activated T helper cells secrete interleukin 2 (IL 2). IL 2 stimulates activated CTLp to proliferate and to differentiate to competent cytotoxic T cells (2). However, investigators from several laboratories have reported that IL 2 alone does not induce the differentiation of CTLp to mature CTL (3–12). We previously described a model system for CTLp differentiation involving at least two other lymphokines *and* IL 2 with thymic T cells used as the lymphokine responder population. Most murine thymocytes 1) are immunologically immature (13, 14), 2) are cortisone sensitive, 3) bind peanut agglutinin (PNA), 4) express high levels of Thy-1 and TL antigens on their surface, and 5) are Lyt-1$^+$2$^+$3$^+$ (15, 16). The cortisone-resistant subset of small thymocytes (5 to 10%), however, resembles mature peripheral T cells functionally and phenotypically (17). These cells 1) are not agglutinated by PNA (14, 18), 2) exhibit marked proliferation in the presence of mitogen and either IL 1 or IL 2 (6, 19), and 3) are stimulated by IL 1 alone to produce IL 2 and to undergo significant proliferation (19). In contrast, the PNA$^+$ thymocytes do not produce IL 2 upon stimulation with IL 1 and mitogen and are therefore not the target cells for IL 1-mediated responsiveness (19).

We reinvestigated CTLp differentiation by using limiting numbers of unfractionated thymic responder cells in help-deficient cultures. Our experiments showed that in addition to IL 2, the generation of cytotoxic responses by PNA$^-$ thymocytes was strictly dependent on the presence of T cell cytotoxicity-inducing factor (TCF1) (7). TCF1 could not be replaced by IL 1 or interferon-γ (IFN-γ). However, proliferation of PNA$^-$ thymocytes in the presence of IL 2 was induced by TCF1 as well as by IL 1. Most important, stimulation of thymocytes with IL 2 plus TCF1 induced expression of IL 2 receptors. These data reinforce the role of TCF1 as an essential lymphokine for the early induction of CTLp differentiation.

MATERIALS AND METHODS

Mice. Male C3H/HeJ mice were purchased from The Jackson Laboratory, Bar Harbor, ME. C3H/Tif were bred in our own colony. All animals were used at 4 to 9 wk of age.

Culture medium. The culture medium used was RPMI 1640 (GIBCO; powdered media) with L-glutamine (2×10^{-3} M) and supplemented with 10% heat-inactivated fetal calf serum (FCS), HEPES (0.02 M), 2-mercaptoethanol (4×10^{-5} M), and gentamicin (50 μg/ml).

Lymphokine preparations. Concanavalin A- (Con A; Pharmacia, Uppsala, Sweden) induced supernatants were prepared by conventional methods. Single cell suspensions from mouse spleens were adjusted to 10^7 cells/ml in culture medium and incubated for 24 hr with 5 μg Con A/ml in humidified air plus 5% CO_2. All preparations were stored at 4°C.

Lymphokine preparations prepared by stimulation with a Con A pulse. Spleen cells (10^7/ml) were incubated in culture medium containing 10 μg Con A/ml. After 5 to 6 hr the cells were washed three times with culture medium. The cells were readjusted to 10^7/ml and cultured in Con A-free culture medium for 24 hr at 37°C in humidified air plus 5% CO_2. The cellfree supernatants contained less than 0.01 μg/ml Con A as measured by using radiolabeled Con A (Amersham, U. K.).

Absorption of IL 2. Con A-induced supernatants were depleted of IL 2 by absorption with cells from an IL 2 receptor bearing long-term T cell line (W-2). One milliliter of Con A-induced supernatant was added to 10^5 W-2 cells (7) and incubated for 48 hr at 37°C. The cellfree supernatant was dialyzed against culture medium and again

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[2] Abbreviations used in this paper: TCF1, T cell cytotoxicity-inducing factor 1; PNA, peanut agglutinin; CTL, cytotoxic T lymphocyte; CTLp, CTL precursor; PMA, phorbol myristic acetate.

incubated for 48 hr with 10^5 W-2 cells per milliliter. The supernatant was again dialyzed against culture medium and contained less than 0.01 U/ml of IL 2 activity.

Preparation of TCF1 from T cell hybridoma cells. K15 T hybridoma cells (20) were suspended at a density of 10^6 cells/ml in serum-free medium, and Con A (Serva, Heidelberg, F. R. G.) was added to a final concentration of 20 μg/ml. The suspension was distributed into 5-ml polypropylene tubes at 1 ml and was incubated for 4 hr at 37°C. The cells were then washed three times, and the supernatants were removed by aspiration. The cells were resuspended in 1 ml of serum-free medium and incubated for 24 hr in humidified air plus 5% CO_2. The supernatants were then harvested, and FCS was added to a final concentration of 10%. They were filtered and stored at 4°C. The activity was stable for at least 2 mo.

Preparation of IL 2. Partially purified IL 2 was prepared as described by Hilfiker et al. (21). EL-4 thymoma cells were suspended at 10^6/ml in RPMI 1640 containing 0.5% heat-inactivated FCS. Ten nanograms per milliliter of phorbol myristic acetate (PMA) were added, and the cells were incubated for 48 hr. The cellfree supernatant was concentrated on an Amicon YM10 membrane. Proteins were precipitated with ammonium sulfate (80% saturation). The precipitate was washed once and dissolved in water. After equilibration with 0.8 M ammonium sulfate in 0.01 M phosphate buffer, the sample was applied on a phenyl-Sepharose column (Pharmacia). The column was washed with 2 vol of 0.64 M ammonium sulfate plus 10% ethanediol. The IL 2-containing peak was finally eluted with 40% ethanediol in 0.16 M ammonium sulfate, concentrated on an Amicon PM10 membrane and equilibrated with column buffer (0.05 M Tris-hydroxy-methylacetate, 0.1 M sodium chloride, pH 7.2, containing 0.05% polyethylene glycol 6000). The material was then applied on a Sephadex G-100 column, and IL 2 activity eluted with an apparent m.w. of 32,000. Peak fractions were pooled and dialyzed against culture medium after adding FCS to a final concentration of 10%.

Assay for IL 1 activity. IL 1 activity was measured in the C3H/HeJ mouse thymocyte assay (22). One unit of IL 1 per milliliter induced 50% of the maximal thymocyte proliferative response.

Preparation of IL 1. IL 1 was prepared from $P388D_1$ cells by following the superinduction protocol of Mizel et al. (23). Briefly, 2×10^6 adherent $P388D_1$ cells were cultured in medium containing 10 μg/ml PMA and 10 μg/ml cycloheximide for 4 hr. This medium was replaced by fresh medium containing 10 μg/ml actinomycin D. After 1 hr the cells were washed three times and were cultured overnight in fresh medium. Cellfree supernatant was collected, and protein was precipitated with ammonium sulfate (65% saturation). After extensive dialysis the sample was applied on a Sephadex G-100 column. IL 1 activity eluted in one peak at 15,000 daltons. The active fractions were pooled, dialyzed, sterilized by filtration, and kept at −20°C. Purified IL 1 preparations were generously provided by Dr. S. Mizel. They were prepared from superinduced $P388D_1$ cells by gel filtration and immunoaffinity chromatography on goat anti-IL 1 antibody coupled to Sepharose 4B (23).

Preparation of anti-IL 1 antibodies. Goat anti-IL 1 antibodies were also generously provided by Dr. S. Mizel (23). The IgG fraction was isolated on DEAE cellulose ion-exchange chromatography after ammonium sulfate precipitation. The protein concentration was adjusted to 5 to 10 mg/ml.

Assay for IL 2 activity. IL 2 in supernatants was measured by the ability to support growth of the IL 2-dependent cell line W-2 (7). Thymidine incorporation after a 20-hr incubation and a 4-hr pulse with 1.0 μCi of [methyl-^{3}H]thymidine (^{3}H-TdR; sp. act. 50 Ci/mmol; Amersham International Ltd., Amersham) was determined. Results are expressed as laboratory units as described by Farrar et al. (1).

Assay for IFN activity. Interferon (IFN) titrations were performed in microtiter wells. L929 cells in 0.2 ml of RPMI 1640 + 5% FCS per well were challenged with vesicular stomatitis virus (Indiana strain). One unit was defined as the minimal amount of IFN capable of conferring protection to 50% of the cells. One microtiter unit per 0.2 ml corresponds to 2 NIH reference units. All titers are expressed in laboratory units.

T cell cytotoxicity assay. Thymic responder cells were co-cultured in a final volume of 0.16 ml medium and varying amounts of lymphokine preparations with 3×10^5 glutaraldehyde-fixed, TNP-haptenated syngeneic spleen cells as stimulators. Cytotoxic activity was measured on day 5 in a 4-hr ^{51}Cr-release test by using 5×10^3 labeled TNP-modified tumor target cells (L929 mouse fibrosarcoma). The data are presented as specific ^{51}Cr release (experimental release − spontaneous release)/(total release − spontaneous release) × 100. Spontaneous release was determined in supernatants of assay wells to which no lymphokine preparations were added. Data are the mean of triplicate cultures. The standard error of the mean was always less than 2.5%.

Thymocyte proliferation. Thymocytes (1 to 3×10^5 per culture) from C3H/HeJ mice were cultured in 0.2 ml medium for 3 days. Varying amounts of IL 2, IL 1, IFN-γ, or TCF1 preparations were added to these cultures. ^{3}H-TdR uptake was measured in a 5- to 12-hr pulse, and results are expressed in cpm of duplicate or triplicate cultures.The C3H/HeJ thymic responder cells used in these assays did not respond to LPS (data not shown).

Separation of PNA$^+$ and PNA$^-$ thymocytes by fluorescence-activated cell sorting. Thymocytes were separated after staining with subagglutinating concentrations of PNA-fluorescein isothiocyanate (PNA-FITC) (24). PNA-FITC at a concentration of 5 μg lectin/ml medium was used for staining 10^7 cells/0.25 ml for 30 min at 4°C. Without washing, the cells were diluted to 5×10^6 cells/ml for sorting in a fluorescence-activated cell sorter (Ortho Diagnostic System). The cells in channels 0 through 32 were collected as the PNA$^-$ fraction and in channels 38 through 100 as the PNA$^+$ fraction. After sorting, samples of fractionated thymocytes were reanalyzed to assess purity. The sorted cell fractions were washed, then resuspended in culture medium, and viable cells were counted in a hemacytometer.

IL 2 receptor determination. Monoclonal antibodies (AMT 13) from a rat hybridoma cell line directed against the murine IL 2 receptor were produced as described (25). Glutaraldehyde-fixed sheep erythrocytes (5×10^6) were added to triplicate cultures of 4×10^5 thymocytes, and the cells were kept on ice for 30 min with or without 100 μl culture supernatant containing AMT 13 antibodies. After another incubation for 60 min on ice with ^{125}I-labeled F(ab')$_2$ fragments of a sheep anti-rat IgG (100 μl of a 1/10 diluted stock with specific activity of 12 μCi/μg; Amersham International Ltd.), the cells were washed; specific binding was determined and expressed as cpm.

Recombinant murine IFN-γ (produced by Genentech, Inc.) was generously supplied by Boehringer Ingelheim, and recombinant human IL 2 (2×10^5 U/ml) was obtained from Cetus Corp. The material was 98% pure and contained 0.02 ng/ml endotoxin.

RESULTS

Requirement for TCF1 plus IL 2 for induction of CTL responses. Investigators from several laboratories have reported that other lymphokines in addition to IL 2 are required for the induction of cytotoxic responses in lymphocyte cultures (3–12). In our system TCF1 was shown to be essential for the induction of cytotoxic responses. One hundred thousand thymic responder cells, metabolically inactive stimulator cells and an excess IL 2 (>40 U/culture) were used (7) (Figure 1). IL 1 or IFN-γ preparations did not substitute for TCF1 under these assay conditions. Control experiments with ^{3}H-labeled Con A showed that Con A that might have been carried over from TCF1 preparations was not responsible for this effect (data not shown).

Induction of thymocyte proliferation with IL 2 plus either TCF1 or IL 1. TCF1 preparations from Con A-pulsed spleen or K15 hybridoma cells were tested for induction of IL 2-responsiveness in C3H/HeJ thymocytes. Substantial proliferation was induced with TCF1 in the absence of stimulator cells or mitogen when 10^5 thymocytes were provided with saturating amounts of IL 2 (100 U/ml) (Figure 2). Likewise, IL 1 preparations induced thymocytes to utilize IL 2 added to the cultures for proliferation. The C3H/HeJ thymic responder cells used in all proliferation assays were LPS unresponsive, as ascertained in control experiments (data not shown).

TCF1 preparations from Con-A pulsed spleen cells contained IFN-γ. Therefore, recombinant IFN-γ was tested for induction of proliferative responses in the presence of IL 2. IFN-γ, however, in concentrations from 4 to 286 U, did not induce IL 2-dependent proliferation (Table I). Thus, both IL 1 and TCF1 induced IL 2-dependent thymocyte proliferation.

Thymocyte target cells for TCF1-induced IL 2-dependent cytotoxicity and proliferation. C3H/HeJ thy-

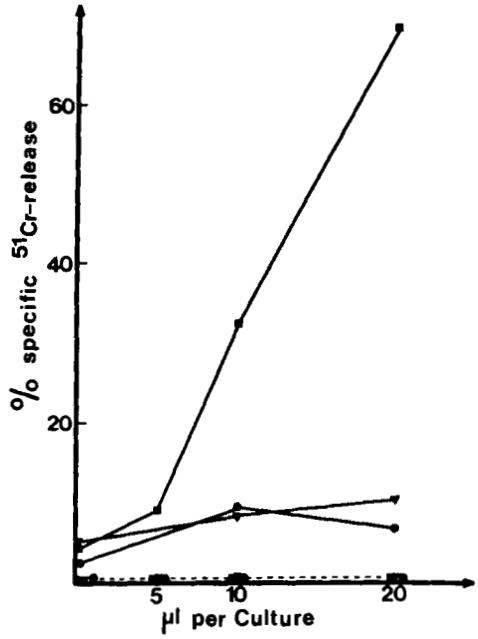

Figure 1. Requirement of TCF1 in excess IL 2 for the induction of cytotoxic responses of thymocytes. Cytotoxic responses were measured in cultures that contained TCF1 (■), recombinant IFN-γ (160 U/ml) (▲), or purified IL 1 (40 U/ml) (●) without (--) or in combination with 20 μl of recombinant IL 2 (400 U/ml) (—).

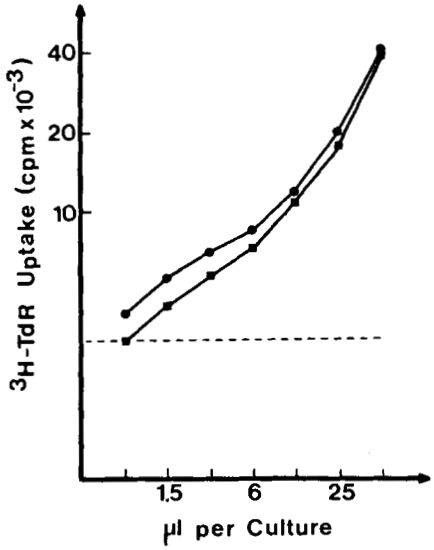

Figure 2. Induction of thymocyte proliferation with TCF1 and IL 2. Proliferation of 5 × 10^5 C3H/HeJ thymocytes induced with either TCF1 from K15 T hybridoma cells (■) or purified IL 1 (2 U/ml) (●) in the presence of 50 μl of recombinant IL 2 (100 U/ml). ^{3}H-TdR uptake with TCF1 or IL 1 alone, <500 cpm; ^{3}H-TdR uptake with IL 2 alone, 4240 cpm.

mocytes were stained with subagglutinating doses of PNA-FITC and separated with a cell sorter into a PNA$^+$ and a PNA$^-$ fraction (24). Reanalysis of the viable sorted thymocytes showed >95% PNA$^-$ cells with a mean fluorescence intensity of 46 and >97% PNA$^+$ cells with a mean fluorescence intensity of 533, respectively. The data in Table II show that TCF1-induced cytotoxicity as well as proliferation in presence of IL 2 is only seen with the PNA$^-$ fraction of the separated thymocytes. Comparable cytotoxic responses were found with one-fourth the number of PNA$^-$ cells compared with unfractionated thymocytes. The PNA$^+$ cells did not respond to the indicated stimuli with cytotoxicity or proliferation. IL 2 alone was able to induce a proliferative response in the PNA$^-$ population. However, this response was small (21%) compared with the synergistic effect of TCF1 and IL 2 and might be due to some preactivated cells.

TABLE I

Lack of synergy of IFN-γ and IL 2 in induction of thymocyte proliferation[a]

Addition to Cultures	IL 2 (U/ml)		
	0	20	40
Medium	100	560	760
IFN 280 U/ml	110	780	640
140 U/ml	100	690	990
70 U/ml	140	940	730
35 U/ml	110	780	800
17 U/ml	110	490	520
9 U/ml	60	760	840
4 U/ml	60	390	910
TCF1	130	8,330	10,120

[a] C3H/HeJ thymocytes (2 × 10^5) were cultured in the presence of various amounts of partially purified IL 2 in 0.2 ml volume. Various amounts of recombinant IFN-γ or a TCF1 preparation from Con A-pulsed spleen cells (50 μl) were added to the cultures. ^{3}H-TdR uptake was measured in a 5-hr pulse on day 3. Data are cpm.

TABLE II

TCF1-induced proliferation and cytotoxicity of thymic subpopulations

Addition to Cultures[a]	Thymocytes					
	^{3}H-TdR Uptake (cpm × 10^{-3})			% Specific ^{51}Cr Release		
	Unsorted	PNA$^-$	PNA$^+$	Unsorted	PNA$^-$	PNA$^+$
IL 2	7.7	16.6	1.2	0.0	0.0[c]	0.0
TCF1	26.4	1.5	2.0	0.6	0.0	0.0
Con A	1.2	NT[b]	0.8	NT	NT	NT
IL 2 + TCF1	70.3	79.4	2.9	15.5	19.6	0.0
IL 2 + Con A	85.8	141.8	2.3	NT	NT	NT

[a] Cultures of 10^5 unseparated or PNA$^-$ or PNA$^+$ C3H/HeJ thymocytes contained recombinant human IL 2 (20 U/ml) and/or TCF1 from K15 cells (25 μl) and/or Con A (1 μg/ml).

[b] NT = not tested.

[c] PNA$^-$ thymocytes (2.5 × 10^4) were cultured for induction of cytotoxicity.

Distinction of TCF1- from IL 1-induced thymocyte proliferation. Both IL 1 and TCF1 induced thymocyte proliferation in the presence of IL 2 (see Figure 2). We used the above lymphokine preparations together with the T cell mitogen Con A to induce thymocyte proliferation. Figure 3 shows that the TCF1 dose-response curve paralleled the dose response of the IL 1 preparation. Thus both purified IL 1 (from macrophages and from the cell line P388D$_1$) and TCF1 (from T hybridoma K15) stimulated thymocyte proliferation in the presence of suboptimal amounts of mitogen (Con A).

Antibodies directed against IL 1 were able to distinguish between the two cytokines (Table III). IL 1-induced but not TCF1-induced proliferation was inhibited (80% and 94%) with a 1/10 dilution of the anti-IL 1 antiserum.

We have preliminary evidence that IL 1 and TCF1 can also be distinguished with sugars as inhibitors of TCF1-induced proliferation and cytotoxicity (to be published).

TCF1 induces IL 2 receptor expression in the presence of IL 2. Induction of IL 2 receptor expression could be the reason for the TCF1-induced IL 2-dependent proliferative response of thymocytes. Monoclonal antibodies (AMT 13) directed against the murine IL 2 receptor (24) were utilized to measure the amount of IL 2 receptor on the cell surface. Table IV shows that AMT 13-binding was greater when cells were cultured with TCF1 plus IL 2 than when cultured with IL 2, TCF1, or IFN-γ alone. Addition of IL 2 shortly before the assays did not increase binding of AMT 13 to the IL 2 receptor (data not shown).

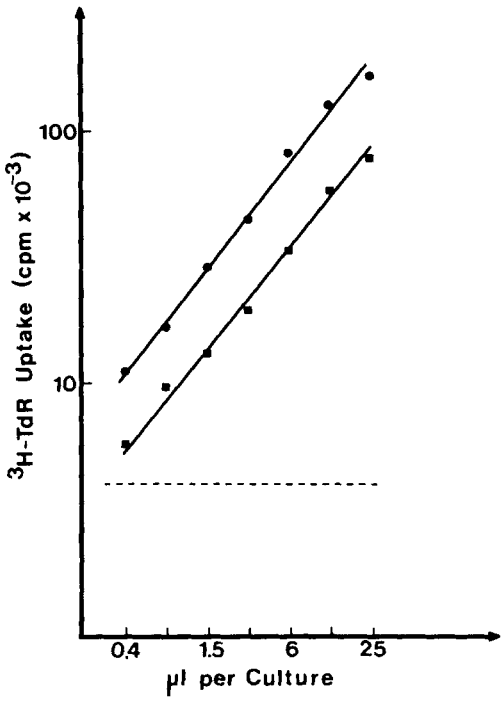

Figure 3. Synergy of TCF1 and IL 1 with Con A in induction of thymocyte proliferation. C3H/HeJ thymocytes (5×10^5) were cultured with or without Con A (1 µg/ml) in 0.2 ml medium. The cultures contained serial twofold dilutions of either IL 1 (from P388D$_1$) (●) or TCF1 (from the T cell hybridoma K15) (■). ^{3}H-TdR uptake was measured in a 5-hr pulse on day 3.

TABLE III

Effect of anti-IL 1 antibodies on IL 1- and TCF1-induced thymocyte proliferation[a]

Addition to Cultures	Dilution of Anti-IL 1 Antibodies			
	—	1/1000	1/100	1/10
a) IL 1	171.6	156.4 (9)	100.7 (41)	10.5 (94)
b) IL 1	154.0	158.4 (0)	117.3 (24)	30.5 (80)
TCF1	231.0	207.4 (10)	217.8 (10)	200.2 (13)

[a] C3H/HeJ thymocytes (3×10^5) were cultured in the presence of recombinant human IL 2 (20 U/ml) in 0.2 ml medium. The cultures contained purified IL 1 (a) (2 U/ml), partially purified IL 1 (b) (2 U/ml), or TCF1 from K15 T hybridoma cells (25 µl) and/or various concentrations of anti-IL 1 antibodies. ^{3}H-TdR uptake was measured in a 10-hr pulse on day 3. Values are cpm $\times 10^{-3}$; numbers in parentheses are percent of inhibition.

TABLE IV

Induction of IL 2 receptor expression on thymocytes

Addition to Cultures[a]	AMT 13 Binding[b] (cpm)
Medium	2,250
IL 2	5,480
IFN-γ	1,590
TCF1	7,880
IFN-γ + IL 2	4,470
TCF1 + IL 2	20,780
Con A supernatant	145,680

[a] Forty-eight-hour cultures of 2×10^6 thymocytes contained recombinant IFN-γ (140 U/ml), TCF1 from K15 T hybridoma cells (10%), and/or partially purified IL 2 (40 U/ml) or Con A supernatant (30%).

[b] Specific binding of monoclonal antibodies (AMT 13) (25) directed against the murine IL 2 receptor was measured on 4×10^5 cells per sample.

DISCUSSION

We recently described the synergistic effect of purified IL 1 and recombinant IL 2 in the induction of IL 2 receptor expression on thymocytes (26). In this report, we present data suggesting that TCF1 has similar effects on thymocytes. TCF1 activity is characterized by its ability to induce cytotoxic responses of thymocytes in the presence of IL 2 (7). The activities of IL 1 and TCF1 differ in two respects: 1) TCF1, but not IL 1, shows a synergistic helper effect in the development of CTL from help-deficient thymocyte cultures. 2) A highly specific antibody to IL 1 inhibits the IL 2-dependent proliferative response of thymocytes induced by IL 1 but not by TCF1.

The target cells of TCF1 and IL 1 are the mature PNA$^-$ thymocytes. Whether TCF1 acts directly on medullary thymocytes or whether immature thymocytes from the cortex are recruited into the lymphokine reactive cell pool remains to be studied. It is also unclear how IL 1 and TCF1 induce IL 2 receptor expression in synergy with IL 2. IL 1, TCF1, or IL 2 alone, however, do not induce IL 2 receptor expression. There are technical limitations to these assays. Significant proliferation and cytotoxicity requires 24 to 48 hr of coincubation with TCF1 and IL 2 (8). In the absence of IL 2, most of the cells were dead after that time. It may be that TCF1 is sufficient for IL 2 receptor expression and that IL 2 only guarantees cell survival. With the method used we cannot answer this question. It may be that IL 1 or TCF1 "prime" the responsive T cell for IL 2 receptor expression, which is then brought about by IL 2 itself giving the second signal. This suggests a regulatory role of IL 2 for expression of its own receptor. This hypothesis is supported by the fact that human IL 2 can regulate the expression of the Tac antigen on human T cells (27, 28). Other stimuli that lead to expression of IL 2 receptors are receptor-inducing factor (29, 30) and antigen (31–34). Whether IL 2 receptor induction by factors or antigen are separate events or segments of the mechanism by which this induction is accomplished in vivo remains to be shown. Also, the question remains why two cytokines (IL 1, TCF1) with similar effects on the IL 2 receptor expression exist. It is conceivable that IL 1 and TCF1 stimulate different subsets of PNA$^-$ cells. This would also explain why the combined action of IL 1 and IL 2 leads only to proliferation, whereas the synergistic effect of TCF1 and IL 2 leads to proliferation and cytotoxicity. The simplest, but unproven, explanation would be that IL 1 induces IL 2 responsiveness in helper T cell precursors, whereas TCF1 induces the same effect in CTLp. These questions can only be answered with sufficient quantities of the pure cytokines tested in limiting dilution cultures on clones of thymocyte helper T cells and CTLp.

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