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Immunology

Interleukin 2-dependent and interleukin 2-independent pathways of regulation of thymocyte function by interleukin 6

(T cells/interleukin 1/tumor necrosis factor)

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ABSTRACT **Recombinant human interleukin 6 (IL-6), also termed B-cell-stimulatory factor 2 (BSF-2) or interferon-β_2, was found to stimulate the proliferation of mouse thymocytes costimulated with phytohemagglutinin (PHA). In addition, IL-6 synergistically enhanced the stimulation of thymocyte proliferation by recombinant human interleukin 1 (IL-1) or interleukin 2 (IL-2). Mature thymocytes lacking peanut agglutinin receptor are the main target of IL-6 action. Incubation of thymocytes with IL-6 in the presence of PHA resulted in an increased expression of the IL-2 receptor (IL-2R) as demonstrated by flow cytometry. Monoclonal antibody specific for the p55 chain of the murine IL-2R significantly reduced IL-6-stimulated thymocyte proliferation in the presence of the optimal concentration of PHA. However, the same monoclonal antibody failed to reduce IL-6-driven thymocyte proliferation in the presence of a suboptimal PHA concentration, suggesting that IL-6 stimulates thymocyte proliferation by way of IL-2-dependent and IL-2-independent pathways. These results indicate that, in addition to its earlier demonstrated ability to promote B-cell differentiation and growth, IL-6 also acts as a growth regulator in cells of the T-lymphocyte lineage. IL-6 is emerging as an important regulatory cytokine with multiple actions on immune functions.**

Interleukin 6 (IL-6), also termed B-cell-stimulatory factor 2 (BSF-2), is a lymphokine originally identified as a B-cell differentiation factor capable of inducing the secretion of immunoglobulins in *Staphylococcus aureus* Cowan I-stimulated normal B lymphocytes and a transformed B-cell line (CESS) (1, 2). IL-6 cDNA was isolated from a human T-cell line (TCL-Nal) encoding a 212-amino acid precursor, cleavage of which yields the mature form of the IL-6 molecule consisting of 184 amino acids (3). Nucleotide sequence analysis showed that the human IL-6 gene consists of five exons and four introns and its organization shows a distinct similarity to the G-CSF gene (4). IL-6 is structurally indistinguishable from the 26-kDa protein (5) or so-called interferon-β_2 (6, 7), which had been identified earlier as proteins produced by fibroblasts stimulated with polyinosinate·polycytidylate [poly(I)·poly(C)] in the presence of cycloheximide (8, 9). In addition to its ability to induce the final maturation of B cells into antibody-forming cells, IL-6 was shown to function as a potent growth factor for murine B-cell hybridomas and plasmacytomas (10) and for Epstein–Barr virus-transformed human B cells (11). IL-6 was also found to be a hepatocyte-stimulating factor regulating the acute-phase protein response in liver cells (12, 13). More recently, it was found that IL-6 is involved in the differentiation of cytotoxic T lymphocytes (14) and regulation of T-cell proliferation (15).

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In the present study, we demonstrate that recombinant human IL-6 can function as a growth factor for activated murine thymocytes. Mature thymocyte population lacking the peanut agglutinin (PNA) receptor was the main target of IL-6 action. Furthermore, we show that treatment of thymocytes with IL-6 along with interleukin 1 (IL-1) or interleukin 2 (IL-2) resulted in a synergistic stimulation of thymocyte proliferation. Treatment of thymocytes with IL-6 also enhanced IL-2 receptor (IL-2R) expression (p55 chain). However, antibody to the p55 chain of the IL-2R blocked only partially (and under some conditions, not at all) the stimulation of thymocyte proliferation by IL-6, indicating that an IL-2-independent mechanism also contributes to the stimulation. Our results provide another example of overlapping biologic activities displayed by structurally unrelated cytokines.

MATERIALS AND METHODS

Cytokines and Antibodies. Purified recombinant human IL-6 (specific activity, 5×10^6 units/mg) was derived from *Escherichia coli* as described (16). Recombinant *E. coli*-derived human IL-1α (specific activity, 3×10^7 units/mg) was kindly provided by Alvin Stern and Peter Lomedico (Hoffmann-LaRoche). Recombinant *E. coli*-derived human IL-2, purified to ≥98% as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis, was kindly supplied by Richard J. Robb (DuPont, Glenolden, PA). Recombinant *E. coli*-derived murine tumor necrosis factor (TNF) (specific activity, 6×10^6 units/mg) was generously supplied by Masafumi Tsujimoto (Suntory Institute for Biomedical Research, Osaka, Japan). The rat monoclonal antibody AMT-13 (IgG2a) (17) and 7D4 (IgM) (18) are both specific for the mouse p55 chain of the IL-2R. AMT-13 antibody in ascites form was used in blocking experiments, and 7D4 antibody produced by a hybridoma cell line obtained from the American Type Culture Collection was used for flow cytometry analysis.

Thymocyte Proliferation Assay. Thymocyte proliferation was measured in flat-bottomed 96-well tissue culture plates. Thymocytes from C3H/HeJ or C57BL/6 mice at 2–3 months of age (The Jackson Laboratory) were seeded at $5–6 \times 10^5$ cells per well in 0.2 ml of RPMI 1640 medium with 5% fetal bovine serum, 2 mM L-glutamine, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 0.05 mM 2-mercaptoethanol (hereafter, culture medium). Special precautions were taken in excising the thymus to avoid any contamination with blood. Phytohemagglutinin (PHA) (prepared and provided by Joel Oppenheim, New York University School of

Abbreviations: IL-6, interleukin 6; BSF-2, B-cell-stimulatory factor 2; IL-1, interleukin 1; IL-2, interleukin 2; TNF, tumor necrosis factor; PNA, peanut agglutinin; IL-2R, IL-2 receptor; PHA, phytohemagglutinin.

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Table 1. Stimulation of thymocyte proliferation by IL-6 and its synergism with IL-1

IL-6, ng/ml	IL-1, ng/ml	[^{3}H]Thymidine uptake					
		Day 2		Day 3		Day 4	
		cpm	SI	cpm	SI	cpm	SI
0	0	3,064 ± 592	—	1,352 ± 210	—	218 ± 16	—
0.5	0	4,317 ± 322	1.4	2,714 ± 98	2.0	391 ± 108	1.8
5	0	6,586 ± 11	2.2	9,548 ± 492	7.1	2,354 ± 447	10.8
50	0	10,091 ± 1402	3.3	19,099 ± 1830	14.1	9,282 ± 1039	42.6
0	0.5	4,574 ± 1322	1.5	5,765 ± 216	4.3	845 ± 457	3.9
0.5	0.5	6,454 ± 404	2.1	11,077 ± 1790	8.2	3,390 ± 1666	15.6
5	0.5	19,769 ± 1705	6.5	34,941 ± 3943	25.8	19,061 ± 1496	87.4
50	0.5	21,466 ± 1491	7.0	39,053 ± 2043	28.9	20,932 ± 1764	96.0

Thymocytes at 6×10^5 per well were incubated in the presence of PHA at 10 μg/ml for 2, 3, or 4 days. The cultures were pulsed with [^{3}H]thymidine during the last 16 hr of incubation. cpm are expressed as mean ± SD. SI, stimulation index.

Medicine, New York, NY) was added to the cultures. Unless noted otherwise, the thymocyte cultures were incubated at 37°C for 3 days, and 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine (New England Nuclear) in 50 μl of medium was added to each well 16 hr before harvest with the aid of a Skatron cell harvester. Results are expressed as mean [^{3}H]thymidine uptake ± standard deviation of triplicate determinations.

Separation of Thymocyte Subpopulations. Freshly isolated thymocytes were incubated at 1.2×10^8 per ml in phosphate-buffered saline (PBS) with 25 μg of PNA (Sigma) per ml at 37°C for 30 min. The PNA-treated thymocytes were overlaid carefully on the top of 10 ml of fetal bovine serum in a 15-ml conical test tube and agglutinated thymocytes were allowed to sediment at room temperature at $1 \times g$ for 30 min. Clumped cells forming a pellet at the bottom were considered to be PNA$^+$, and nonpelleted cells at the top of the gradient were considered to be PNA$^-$. This procedure yielded a population of PNA$^+$ cells representing 20–30% of the initial thymocytes. After separation, the cells were incubated with 10 mg of D-galactose per ml in culture medium at 37°C for 10 min to dissociate bound PNA from the cells. The PNA$^+$ and PNA$^-$ populations were then washed twice in culture medium before use.

IL-2 Assay. IL-2 activity was quantitated by the stimulation of [^{3}H]thymidine uptake in the murine CTLL-1 cell line kindly provided by Karl Welte (Sloan-Kettering Institute for Cancer Research, New York, NY). Aliquots of 4×10^3 CTLL-1 cells were incubated in 0.2 ml per well of RPMI 1640 medium supplemented with 10% fetal bovine serum and 0.05 mM 2-mercaptoethanol in the presence of 1:2 serial dilutions of test samples in 96-well plates. After a 20-hr incubation, 0.5 μCi of [^{3}H]thymidine in 50 μl of medium was added to each well, and the cells were harvested 4 hr later. IL-2 titers were calculated by probit analysis. The sensitivity of this assay in our hands is ≈0.05 unit/ml of IL-2.

Immunofluorescence Staining and Flow Cytometry Analysis. For indirect immunofluorescence, thymocytes were first incubated with monoclonal antibody 7D4 and, after washing, stained with fluorescein isothiocyanate-conjugated goat anti-rat immunoglobulin. Control samples were stained with the fluorescence conjugate alone. Cell surface fluorescence was quantified by using an Ortho 50H cytofluorograph equipped with a 5-W argon laser. Viable cells were gated on the basis of forward and 90° light scatter. Fluorescence emission was measured by filtering the light passed by a dichroic mirror through a narrow-band green filter (530 ± 10 nm). The data collected from 10^4 cells were analyzed and histograms were generated.

RESULTS

Stimulation of Thymocyte Proliferation by IL-6 and Its Synergism with IL-1 and IL-2. In the presence of the optimal concentration of PHA (10 μg/ml), IL-6 showed a marked, time- and dose-dependent stimulation, with a maximal 14- and 40-fold increase in [^{3}H]thymidine uptake on day 3 and day 4, respectively (Table 1). When lower PHA concentrations were used, stimulation by IL-6 was less marked and no stimulation occurred in the absence of PHA (not shown). Similar results were obtained with thymocytes from C3H/HeJ or C57BL/6 mice, indicating that the stimulation observed was not due to a trace amount of lipopolysaccharide contaminating the IL-6 preparation. In addition, IL-6 synergistically potentiated IL-1-stimulated thymocyte proliferation (Table 1).

A synergistic stimulation of thymocyte proliferation also occurred with IL-2 and IL-6. IL-6, added at 0.5, 5, or 50 ng/ml, significantly augmented [^{3}H]thymidine uptake by IL-2-stimulated thymocytes in the presence of PHA at 10 μg/ml (Table 2). In view of the known role of IL-2 as a mediator of IL-1-induced thymocyte proliferation (19), the synergy between IL-2 and IL-6 (Table 1) also may be the result of an interaction between IL-6 and endogenously produced IL-2.

Mature Thymocytes Lacking PNA Receptor as Main Target Cells of IL-6 Action. To define the target cells of IL-6 action, thymocytes were separated into PNA$^+$ and PNA$^-$ subpopulations and examined for proliferation in response to IL-6. IL-6 caused a marked stimulation in mature thymocytes lacking PNA receptor, indicating that PNA$^-$ thymocytes are the main target cells (Table 3). The slight increase in DNA synthesis of PNA$^+$ thymocytes in response to IL-6 could be due to a small quantity of PNA$^-$ cells contaminating the PNA$^+$ cell population. Immature thymocytes are known to be unresponsive to concanavalin A (Con A) and IL-2 (20).

Enhancement of IL-2R Expression on Thymocytes by IL-6. Potentiation of IL-1- or IL-2-stimulated thymocyte proliferation by IL-6 suggested that IL-6 may affect the interaction between IL-2 and the IL-2R. We therefore examined the effect of IL-6 on IL-2R expression on thymocytes with the aid of flow cytometry, using the 7D4 antibody specific for mouse IL-2R (18). Fig. 1 shows that treatment of thymocytes with

Table 2. Synergism of IL-6 with IL-2 in the stimulation of thymocyte proliferation

IL-6, ng/ml	[^{3}H]Thymidine uptake, cpm		
		IL-2	
	No IL-2	0.03 unit/ml	0.1 unit/ml
0	357 ± 43	683 ± 38	2,448 ± 261
0.5	905 ± 48	1723 ± 343	5,851 ± 161
5	3888 ± 816	6073 ± 968	11,964 ± 1560
50	5945 ± 1066	7835 ± 140	14,623 ± 429

Thymocytes at 6×10^5 per well were incubated in the presence of PHA at 10 μg/ml for 3 days. The cultures were pulsed with [^{3}H]thymidine during the terminal 16 hr of incubation. cpm are expressed as mean ± SD.

Table 3. Stimulation of thymocyte proliferation by IL-6: response of thymocyte subpopulations

Experiment	Thymocyte population	[^{3}H]Thymidine uptake, cpm			
			IL-6		
		No IL-6	0.5 ng/ml	5 ng/ml	50 ng/ml
1	PNA$^-$	234 ± 82	882 ± 95	3588 ± 95	5463 ± 96
	PNA$^+$	166 ± 90	172 ± 81	214 ± 77	247 ± 30
	Unseparated	386 ± 92	555 ± 54	2857 ± 219	5226 ± 571
2	PNA$^-$	674 ± 58	ND	4441 ± 688	9107 ± 911
	PNA$^+$	214 ± 30	ND	302 ± 39	429 ± 76
	Unseparated	495 ± 64	ND	3394 ± 332	7997 ± 1066

Thymocytes at 6×10^5 per well were incubated in the presence of PHA at 10 μg/ml for 3 days. The cultures were pulsed with [^{3}H]thymidine during the terminal 16 hr. cpm are expressed as mean ± SD. ND, not determined.

IL-6 in the presence of PHA for 2 days resulted in an increase in IL-2R expression. The percentage of IL-2R-positive cells among control thymocytes, thymocytes treated with 5 ng of IL-6 per ml, and thymocytes incubated with 50 ng of IL-6 per ml was 5.3%, 14.8%, and 22.8%, respectively. Similar results were obtained when murine thymocytes were incubated with IL-6 for 3 days. IL-6 was previously found to enhance the expression of IL-2R on an IL-2-dependent human T-cell line (21).

Effect of anti-IL-2R Antibody on Thymocyte Proliferation Stimulated by IL-6 and TNF. To examine whether an interaction between IL-2 and the IL-2R is required for IL-6-stimulated thymocyte proliferation, we determined the effect of AMT-13 antibody, known to block the high-affinity mouse IL-2R (17), on thymocyte proliferation. AMT-13 antibody almost completely inhibited IL-2-stimulated proliferation, while displaying no suppression of thymocyte proliferation stimulated by IL-6 in the presence of PHA at 5 μg/ml (Table 4). In the presence of a higher concentration of PHA (10 μg/ml), AMT-13 antibody reduced IL-6-stimulated thymocyte proliferation by ≈50%.

In agreement with Ranges *et al.* (22), we found that murine TNF, but not human TNF (23, 24), stimulated murine thymocyte proliferation. Combined treatment of thymocytes with murine TNF and IL-6 resulted in an additive to synergistic stimulation (data not shown). Unlike IL-6, TNF-stimulated thymocyte proliferation was not affected by AMT-13 antibody (Table 4), indicating that the action of TNF on thymocytes is IL-2-independent.

DISCUSSION

Our results demonstrate that IL-6 can stimulate thymocyte proliferation and IL-2R expression on thymocytes, suggesting a role of IL-6 in T-cell development and function. Stimulation of the growth of thymocytes and mature T cells by murine and human IL-6 was also demonstrated recently by Garman *et al.* (15), Lotz *et al.* (25), and Uyttenhove *et al.* (26). Until now very little was known about the mechanism of IL-6-mediated activation of thymocytes and T cells. Garman *et al.* (15) found that IL-6 enhanced IL-2 production by murine T cells costimulated with Con A, but Uyttenhove *et al.* (26) failed to detect IL-2 activity in supernatants of

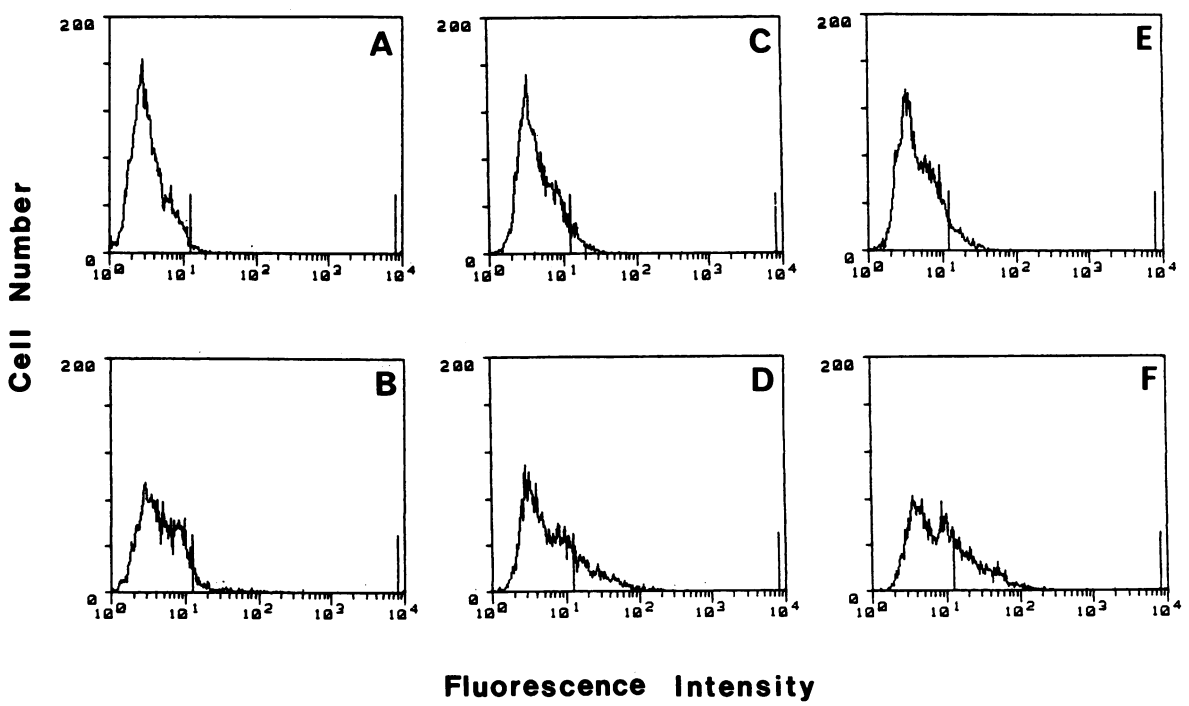

FIG. 1. Induction of IL-2R expression on thymocytes by IL-6. The expression of IL-2R on control thymocytes (*A* and *B*) and thymocytes (4×10^6 per ml) incubated for 2 days with IL-6 at 5 ng/ml (*C* and *D*) or 50 ng/ml (*E* and *F*) in the presence of PHA at 10 μg/ml was assessed with monoclonal antibody 7D4 specific for the IL-2R by indirect immunofluorescence staining and flow cytometry (*B*, *D*, and *F*). Control cells (*A*, *C*, and *E*) were stained with fluorescein isothiocyanate-conjugated second antibody only. % positive cells: *A*, 1.3; *B*, 6.6; *C*, 6.9; *D*, 21.7; *E*, 8.3; *F*, 31.1.

Table 4. Effect of antibody specific for IL-2R on thymocyte proliferation stimulated by IL-6 and TNF

PHA, μg/ml	Antibody*	[^{3}H]Thymidine uptake, cpm			
		No IL-6	IL-6, 50 ng/ml	TNF, 50 ng/ml	IL-2, 0.2 unit/ml
5	None	728 ± 80	2,115 ± 211	2015 ± 335	30,197 ± 2165
	AMT-13 (5 μg/ml)	563 ± 59	1,911 ± 147	1841 ± 41	955 ± 194
	AMT-13 (20 μg/ml)	482 ± 55	1,926 ± 159	1996 ± 214	487 ± 40
	Control antibody	610 ± 76	1,973 ± 300	2021 ± 326	31,430 ± 3134
10	None	1167 ± 156	14,707 ± 1220	3144 ± 353	42,598 ± 1176
	AMT-13 (5 μg/ml)	827 ± 88	5,982 ± 1121	3338 ± 274	1,587 ± 399
	AMT-13 (20 μg/ml)	1070 ± 124	6,516 ± 508	3276 ± 490	1,001 ± 168
	Control antibody	1196 ± 249	12,884 ± 2627	3563 ± 388	39,701 ± 2148

Thymocytes at 6×10^5 per well were incubated in the presence of PHA at 5 or 10 μg/ml for 3 days. The cultures were pulsed with [^{3}H]thymidine during the terminal 16 hr. cpm are expressed as mean ± SD.
*AMT-13 is a monoclonal antibody specific for the murine IL-2R; an unrelated and immunoglobulin type-matched antibody was used as a control.

IL-6-treated murine T cells. Furthermore, Lotz *et al.* (25) failed to detect an increase in the number of IL-2Rs in human thymocytes. Although two groups reported that proliferation of thymocytes or T cells in response to IL-6 and mitogen was reduced by antibodies to the IL-2R (15, 25), these results do not necessarily prove that IL-6 action results in a stimulation of IL-2 production or IL-2R expression. Trace amounts of IL-2 could be produced even in the absence of IL-6 (e.g., due to contamination of thymocytes with T cells from peripheral blood), and the stimulatory action of IL-6 could be partly due to a synergism with endogenous IL-2.

Two lines of evidence presented in this study suggest that the stimulation of thymocyte proliferation by IL-6 depends partly on IL-2/IL-2R interaction. (*i*) We found that IL-6 enhanced IL-2R expression (Fig. 1). (*ii*) We showed that the AMT-13 antibody reduced by about 50% IL-6-driven proliferation of thymocytes in the presence of PHA at 10 μg/ml (Table 4). However, the same antibody failed to affect IL-6-stimulated proliferation in the presence of a suboptimal PHA concentration of 5 μg/ml, suggesting that under the latter conditions proliferation was IL-2-independent. AMT-13 antibody binds to the p55 chain of the murine IL-2R, thereby blocking IL-2 binding to the high-affinity IL-2R (17). It is not known whether the IL-2-dependent and -independent responses are the function of the same cell population or two different thymocyte populations. If two cell populations are involved, both are likely to be PNA^- (mature) thymocytes since no response to IL-6 was seen with PNA^+ thymocytes (Table 3).

Although no IL-2 activity (<0.05 unit/ml) could be detected in the culture fluids of IL-6-stimulated thymocytes (data not shown), indirect evidence supports the conclusion that IL-6 is likely to stimulate the production of small amounts of IL-2 in thymocytes in the presence of PHA at 10 μg/ml. This conclusion is supported by the observation that antibody AMT-13 reduced IL-6-driven proliferation in the presence of PHA at 10 μg/ml but did not reduce significantly proliferation in control or TNF-treated cultures (Table 4). Since TNF is known to potentiate the mitogenic action of IL-2 in murine thymocytes (22), blocking of the IL-2R would be expected to reduce TNF-driven proliferation if effective levels of IL-2 had been present. The absence of an inhibitory action of antibody AMT-13 in TNF-treated cells together with the clear inhibition seen in IL-6-treated cells in the presence of PHA at 10 μg/ml strongly suggest that IL-2 had been generated in the IL-6-treated thymocytes. It is interesting that TNF too was found to increase IL-2R expression on human T lymphocytes (27).

Our data and the results of other recent studies show that in its actions on thymocytes and T cells IL-6 shows many similarities with IL-1. Like IL-1, IL-6 can enhance the expression of IL-2R on a T-cell line (21) and thymocytes (Fig. 1). In addition, IL-1 and IL-6 apparently share the ability to stimulate IL-2 production (15, 19). However, unlike IL-1, IL-6 also acts through an IL-2-independent mechanism, as suggested by our present data. Existence of an IL-2-independent mechanism is likely to be responsible for the synergistic action of IL-6 with IL-1 (Table 1) and IL-2 (Table 2) in stimulating thymocyte proliferation. It is interesting that in fibroblasts IL-1 (10) and TNF (28) stimulate IL-6 production. Whether IL-1 or TNF also stimulates cells of the T-cell lineage to produce IL-6 is not yet known. However, IL-6 is unlikely to mediate TNF-induced thymocyte proliferation in view of the lack of an inhibitory action of the AMT-13 antibody on TNF-stimulated thymocyte proliferation (Table 4). In addition, we found that TNF-induced thymocyte proliferation was much less affected by the PHA concentration employed than IL-6-stimulated proliferation (Table 4 and data not shown).

Another cytokine that can stimulate thymocyte proliferation by an IL-2-independent mechanism is IL-4 (29, 30). However, the mechanism of the IL-2-independent portion of IL-6 action on thymocytes is probably different from that of IL-4. We found that, unlike in the results reported with IL-4 (29, 30), IL-6 failed to stimulate thymocyte proliferation in the presence of phorbol 12-myristate 13-acetate (data not shown). In view of the wide variety of cells that produce IL-6 (i.e., monocytes, T cells, fibroblasts, hepatocytes) and the variety of actions on B-cell and T-cell functions recently associated with IL-6 (see ref. 31 for review), this cytokine is emerging as an important immunoregulatory molecule.

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