Interleukin 2-dependent release of interleukin 3 activity by T4⁺ human T-cell clones

(lymphokines/growth factors)

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ABSTRACT We have isolated and studied two alloreactive, T4⁺, human lymphocyte clones that release interleukin 2 (IL-2) and interleukin 3 (IL-3) bioactivities upon stimulation with IL-2, alloantigen, or Sepharose-conjugated antibodies directed against the T3 protein. Anti-IL-2 receptor monoclonal antibodies block IL-2-, alloantigen-, or anti-T3-stimulated IL-3 release. Hence, release of IL-3 activity in each circumstance is rigorously dependent upon activation of the IL-2 receptor. Even low, nonmitogenic concentrations of recombinant IL-2 stimulated IL-3 release.

The complexity that exists within the T-lymphocyte system with regard to functionally disparate subpopulations of cells has made precise analysis of T-lymphocyte responses at the bulk mixed-lymphocyte reaction level difficult. The use of cloned T cells permits an exacting dissection of the heterogeneity of alloactivated T cells, especially in regard to the mechanisms regulating lymphokine secretion. Murine interleukin 3 (IL-3), a hematopoietic growth and differentiation factor, has been characterized at the biochemical (1-4) and genetic (3-5) level. In the mouse, antigen-activated Lyt-1⁺ T cells have been identified as the source of IL-3 (3, 4, 6). The biological activities of the murine lymphokine have been investigated in detail (7), but little information is available concerning human IL-3 (8). We now describe two alloreactive human T-cell clones that release IL-3 bioactivity upon stimulation with specific antigen, anti-T3 antibody, or IL-2. Further analysis reveals that IL-3 release in response to each of these stimuli is rigorously dependent upon activation of IL-2 receptors. Even low, nonmitogenic concentrations of recombinant IL-2 stimulated IL-3 release.

MATERIALS AND METHODS

Human T-Cell Clones. The human T-cell clones C1-1 and C10-1 were obtained from a single donor's peripheral blood mononuclear cells after an 18-hr mixed-lymphocyte reaction generated against irradiated (5500 rads; 1 rad = 10⁻² J/kg) cells of the Epstein-Barr virus-transformed B-cell line JY (HLA DR4/DR6) (9). Cells harvested after the mixed-lymphocyte reaction were stained with the anti-IL-2-receptor monoclonal antibody (mAb) B1 49.9 (10) by an indirect immunofluorescence technique, and the IL-2 receptor-positive cells were isolated by flow cytometry (FACS 440, Becton Dickinson). These mAb 49.9-positive alloactivated T lymphocytes were then immediately cloned under limiting-dilution conditions (displaying a "single-hit" type slope) in medium containing highly purified, endotoxin-free, *Esche-*

richia coli-derived recombinant IL-2 (rIL-2) (Biogen, Cambridge, MA) and autologous irradiated (5500 rads) feeder cells, in V-bottom microculture plates (Flow Laboratories). After 4 weeks of growth in medium containing rIL-2, the pedigree of T-cell clones was established by phenotypic cell surface markers, and the clones were analyzed for reactivation after confrontation with the original stimulator JY line or other allogeneic B-cell lines. Two selectively alloreactive anti-JY T-cell clones, C1-1 and C10-1, were subcloned at one cell per well in microculture plates containing rIL-2 and autologous feeder cells. These clones were cultured with rIL-2 for several months. Aliquots of the T-cell clones were periodically assessed for surface expression of IL-2 receptors by immunofluorescence staining with the anti-IL-2-receptor mAb 49.9. When a decrease in density of cell surface IL-2 receptors was noted, the clones were stimulated with a Sepharose-linked anti-T3 mAb designated 64.1, using the technique of Meuer *et al.* (11), in order to maintain activity. The clones exhibited stable phenotype, function, and specific antigenic reactivity without further antigenic restimulation (9).

[³H]Thymidine-Incorporation Assays. Assessment of proliferative stimuli was performed in triplicate cultures in 96-well V-bottom microculture plates. Cloned C1-1 or C10-1 cells (5 × 10⁴) were cultured either with 5 × 10³ irradiated (5500 rads) JY (the original stimulator line) or Laz 509 (HLA DR7⁺; a third-party, Epstein-Barr virus-transformed B-cell line) cells, or with medium alone, or with the mitogenic pan-T-cell mAb 64.1 [provided by J.A.H.; recognizes the T3 protein (unpublished data)] conjugated to CNBr-activated Sepharose 4B beads (final bead dilution 1:3000), or with purified rIL-2 (Biogen). After 18 hr, the various cultures were each incubated with 1 μCi (1 Ci = 37 GBq) of [³H]-thymidine (Amersham) and harvested onto fiberglass filters 18 hr later.

Determination of IL-2 and IL-3 Secretion by T-Cell Clones. Culture supernatants were assayed for their ability to support proliferation of the IL-2 dependent murine T-cell line CTLL-2 (a gift of K. A. Smith, Dartmouth Medical School) (12).

Two cloned IL-3-sensitive mouse cell lines, FDC-P1 and DA-1 (a gift of J. Ihle, Frederick Cancer Research Facility, Frederick, MD) (13), were each cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum and 25% (vol/vol) IL-3-rich supernatants from cultured WEHI-3 cells.

Abbreviations: CSF, colony-stimulating factor; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; r, recombinant.

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Culture supernatants to be assayed for IL-3 bioactivity were serially diluted in medium and incubated with 5×10^4 FDC-P1 or DA-1 cells in a total volume of 0.2 ml in flat-bottom microtiter plates. After 18 hr of incubation, 1 μ Ci of [3 H]thymidine was added per well, and the cultures were harvested 4 hr later by an automatic harvester. Ultrapure human IL-1 (Genzyme, Boston, MA), purified rIL-2, recombinant human colony-stimulating factor 2 (rCSF-2) (DNAX, Palo Alto, CA), purified recombinant γ -interferon (rIFN- γ , a gift from W. Fiers, Ghent, Belgium), recombinant murine IL-3 (DNAX), and purified rIFN- α (Biogen) were tested in triplicate at multiple concentrations for their capacity to support proliferation of IL-3-sensitive FDC-P1 and DA-1 cell lines (14).

mAbs. mAb Tac (a gift of T. A. Waldmann, National Institutes of Health) (15, 16) and the mAb B1 49.9 (49.9) (Laboratoire Immunotech, Marseilles, France) (10) define the IL-2 receptor. Anti-T1, -T4, -T8, -T3, and -T11 mAbs were the gift of E. Reinherz (Dana-Farber Cancer Institute, Boston, MA). Anti-Leu-11a (Becton Dickinson) recognizes an antigen on the surface of human natural killer cells. Nonspecific mAb binding to cloned T cells was evaluated by interaction with a mAb that reacts with mycoplasma-infected cells (courtesy of M. Hemler, Dana-Farber Cancer Institute). The second antibody used for the indirect immunofluorescence staining was fluorescein-conjugated goat anti-mouse Ig (Tago, Burlingame, CA). Each mAb was used in antibody excess (dilution, 1:100 to 1:500).

RESULTS AND DISCUSSION

Precise elucidation of the functional activities exhibited by individual alloantigen-responsive T cells obviously requires the availability of clonal populations. Clones were generated by cultivating IL-2 receptor-positive (i.e., mAb 49.9-positive), alloactivated T cells, isolated from an 18-hr MLR by use of a cell-sorter (see *Materials and Methods* and ref. 9), by culturing the activated T cells in limiting dilutions in medium containing purified rIL-2. Two alloreactive, phenotypically stable clones, C10-1 and C1-1, retained functional properties (Tables 1 and 2) throughout the subcloning procedure. Subcloning was initiated at 1 cell per well in medium containing rIL-2. Both clones are T1 $^+$, T3 $^+$, T4 $^+$, T11 $^+$, and T8 $^-$ and reacted with mAb 49.9 (data not shown). Both alloreactive T-cell clones mounted a proliferative response against the initial stimulator cell line JY but not against the third-party stimulator cell line Laz 509, after 2 days of coculture (Table 1). Clone C1-1 exerted cytotoxicity against the original stimulator cell line JY (Table 2).

Table 1. Proliferative responses of clones C1-1 and C10-1

Stimulus	[3 H]Thymidine incorporation, * cpm
C1-1	
JY cells	4,025
Laz 509 cells	292
rIL-2 †	47,000
None	88
C10-1	
JY cells	27,495
Laz 509 cells	514
mAb 64.1 ‡	14,529
rIL-2 †	15,844
None	242

* Assayed by 18-hr incubation with the stimulus, followed by addition of [3 H]thymidine and further incubation for 18 hr. Assays done in triplicate (SD $\leq$ 10% of the mean).

† rIL-2 was added to the culture medium at a final dilution of 1:20,000.

‡ Coupled to Sepharose beads and added to the culture medium at a final dilution of 1:300.

Table 2. Cytotoxicity analysis of clones C1-1 and C10-1

Target cells	% specific cytotoxicity* with	
	C1-1 effectors	C10-1 effectors
JY	76	0
Laz 509	0	0
K562 †	0	2

* Cytotoxicity was assessed in a standard 4-hr cell-mediated 51 Cr-release assay. % specific lysis was calculated as [(test cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)] $\times$ 100. Effector/target ratio was 10:1. Assays were done in triplicate (SD $\leq$ 10% of the mean).

† A human myelocytic cell line used to test natural killer activity.

Recombinant CSF-2, purified rIL-2, rIFN- γ , rIFN- α , and human IL-1 failed to support proliferation of the IL-3 indicator cell line DA-1 (Table 3). Thus, the described IL-3 bioactivity released by the T-cell clones is not due to IL-1, IL-2, CSF-2, or IFNs. Growth of the FDC-P1 line was supported by IL-3 or CSF-2 but not IL-1, IL-2, or IFNs. Hence, the DA-1 line is apparently a more selective IL-3 indicator line than the FDC-P1 line, which is sensitive to both IL-3 and CSF-2. The responsiveness of the FDC-P1 line to both murine CSF-2 and IL-3 has been observed by others (17). The IL-3 bioactivity described hereafter was defined by use of the DA-1 indicator line. When each of the T-cell clones was restimulated with the original stimulator cell line, but not when they were stimulated with a third-party stimulator cell line, IL-2 (Fig. 1) and IL-3 (Fig. 2) bioactivities were detectable in the culture supernatants after 2 days of coculture. Culture supernatants of the stimulator cell lines JY and Laz 509 did not contain IL-2 or IL-3 activities. These data do not discriminate among the possibilities that IL-3 release can be directly triggered by activation of the T-cell antigen receptor-T3 complex, by IL-2, or by both. Hence, purified rIL-2 was incubated with the T-cell clones, inducing both [3 H]thymidine incorporation and IL-3 release (Fig. 2A). Interestingly, Sepharose-bound mAb 64.1 (recognizes T3 protein associated with T-cell antigen receptor) induced both proliferation of these T-cell clones and release of IL-3 (Fig. 2B). Again these data show an association between IL-2 and IL-3 release. Anti-T3 mAb coupled to Sepharose beads has been shown (11) to induce proliferation of T-cell clones and to trigger IL-2 release. Either anti-IL-2 receptor mAb 49.9 (Fig. 3A) or Tac (Fig. 3B), which block IL-2 binding to its receptor, inhibited rIL-2-driven [3 H]thymidine incorporation and IL-3 release. IL-3 release stimulated by rIL-2 was not inhibited by anti-T1 or anti-T4 mAb (Fig. 3A and B). After incubation of clone C10-1 with JY cells, the anti-IL-2 receptor mAb Tac also blocked the release of IL-3 (Fig. 3C) but not IL-2 (data not shown). In contrast, anti-T1 mAb, which binds to T-cells at a site distinct from the IL-2 receptor, does not

Table 3. Response of IL-3-sensitive indicator lines to cytokines

Stimulus	[3 H]Thymidine incorporation, cpm (mean $\pm$ SD)	
	DA-1	FDC-P1
Medium (control)	4,376 $\pm$ 215	3,685 $\pm$ 192
WEHI-3 supernatant	61,298 $\pm$ 1321	49,666 $\pm$ 928
IL-1	4,652 $\pm$ 279	3,524 $\pm$ 283
rIL-2	4,273 $\pm$ 351	3,869 $\pm$ 386
rIL-3	48,915 $\pm$ 1127	50,183 $\pm$ 1576
rCSF-2	5,145 $\pm$ 541	44,381 $\pm$ 1467
rIFN- γ	4,162 $\pm$ 201	3,456 $\pm$ 215
rIFN- α	4,510 $\pm$ 189	3,613 $\pm$ 385

Each cytokine was tested at multiple concentrations, using doses known to have optimal bioactivity in other test systems (unpublished data).

FIG. 1. IL-2 secretion by T-cell clones. (A) IL-2 bioactivity in supernatants of 2-day cultures of C1-1 (▲), C1-1 plus JY (■), or C1-1 plus Laz 509 (◆). (B) IL-2 bioactivity in supernatants of 2-day cultures of C10-1 (▲), C10-1 plus JY (■), or C10-1 plus Laz 509 (◆). $[^3\text{H}]$ thymidine incorporation of the indicator cell line CTLL-2 is plotted versus the dilution of the supernatants in the medium of the CTLL-2 cells.

block alloantigen-stimulated IL-3 release (Fig. 3C). Moreover, release of IL-3 by the T4^+ clones stimulated by Sepharose-conjugated anti-T3 mAb 64.1 was blocked by the anti-IL-2 receptor mAb 49.9, but not by the anti-T4 mAb (data not shown). As the concentration of exogenous rIL-2 is decreased (Fig. 3D), a concomitant decrease in released IL-3 activity is observed (Fig. 3E), demonstrating that IL-2-stimulated IL-3 release is dose-dependent. Interestingly, submitogenic concentrations of rIL-2 did evoke IL-3 release from the T-cell clones (Fig. 3D and E).

The present report precisely defines a stimulus for IL-3 secretion by these human T4^+ lymphocyte clones. IL-3 is a product of activated murine helper T cells (2-4, 6, 14). For example, Guerne *et al.* (6) demonstrated that the vast majority of high IL-3 producers were found among the Lyt-2^- T-cell population in the mouse. Certain cloned antigen-specific T cells in the mouse also produce IL-3 after stimulation with antigen (14). Miller and Stutman (18) showed that almost all murine T cells that produce IL-3 also release IL-2 activity, and they suggested that the regulatory events that control IL-2 generation among allostimulated helper cells

FIG. 2. IL-3 secretion by T-cell clones. (A) IL-3 bioactivity in supernatants of 2-day cultures of C1-1 (▲), C1-1 plus JY (■), C1-1 plus rIL-2 (dilution, 1:20,000) (●), or C1-1 plus Laz 509 (◆). (B) IL-3 bioactivity in supernatants of 2-day cultures of C10-1 (◆), C10-1 plus JY (■), C10-1 plus rIL-2 (dilution, 1:20,000) (●), C10-1 plus mAb 64.1-Sepharose (dilution 1:300) (*), or C10-1 plus Laz 509 (▲). $[^3\text{H}]$ thymidine incorporation of the indicator cell line DA-1 is plotted versus the dilution of the supernatants in the medium of the DA-1 cells.

also control IL-3 levels. Our results support these observations and show that IL-3 activity release is directly stimulated by interaction of IL-2 with IL-2 receptors. The human T-cell clones described herein express IL-2 receptors and are highly reactive to rIL-2. We doubt the recent contention by Palacios (8) that lectin-activated, IL-3-producing human T cells lack IL-2 receptors, as IL-3 release from our T-cell clones is rigorously dependent upon activation of the IL-2 receptor. Purified rIL-2 stimulates IL-3 release. IL-3 release stimulated by rIL-2, alloantigen, or anti-T3 mAb is blocked by occupation of the IL-2 receptor by the anti-IL-2-receptor mAbs 49.9 or Tac. Hence, alloantigen-driven IL-3 release is caused by IL-2 release. The role of IL-2 in stimulating IL-3 release follows observations in other laboratories that IL-2 induces the release of B-cell growth factor (19) and $\text{IFN-}\gamma$ (20). The data reported here further document the central role of IL-2 in T-cell activation.

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FIG. 3. IL-2-dependent release of IL-3 activity.

(Left) IL-2 receptor blockade inhibits IL-3 release. (A) Cells (5×10^4 per well in 96-well microculture plates) of clone C10-1 were incubated for 1 hr at room temperature either in 50 μ l of RPMI 1640 supplemented with 10% normal human serum (■) or in 50 μ l of the same medium but containing 0.01 mg of anti-T4 mAb 49.9 (◆) or of mAb 49.9 (▲) per ml; 150 μ l of culture medium containing rIL-2 at a 1:10,000 dilution was then added to each well and the cultures were incubated for 2 days at 37°C in a 5% CO₂ atmosphere. IL-3 bioactivity in culture supernatants then was tested by using the indicator cell line DA-1. Proliferation of clone C10-1 also was assessed by [³H]thymidine incorporation: C10-1 plus rIL-2, 34,000 cpm; C10-1 plus anti-T4 mAb plus rIL-2, 14,500 cpm; C10-1 plus mAb 49.9 plus rIL-2, 800 cpm. (B) C10-1 cells were treated as in A except that the 1-hr room-temperature incubation was either without mAb (●) or with a 1:300 dilution of highly purified anti-T1 mAb (□) or a 1:100 dilution of highly purified Tac mAb (▲). IL-3 bioactivity in culture supernatants was tested as in A. Proliferation of C10-1 cells also was assessed by [³H]thymidine incorporation: C10-1 plus rIL-2, 9132 cpm; C10-1 plus anti-T1 mAb plus rIL-2, 8469 cpm; C10-1 plus Tac mAb plus rIL-2, 1755 cpm. (C) C10-1 cells (5×10^4 per well) were cocultured for 2 days with irradiated JY cells (2.5×10^4 per well) in RPMI 1640 medium supplemented with 10% normal human serum; after 2 days further incubation, either without mAb (■) or with a 1:300 dilution of anti-T1 mAb (□) or Tac mAb (▲), IL-3 bioactivity was tested as in A. Proliferation of C10-1 cells also was assessed by [³H]thymidine incorporation: C10-1 plus JY, 5983 cpm; C10-1 plus JY plus anti-T1 mAb 5543 cpm; C10-1 plus JY plus Tac mAb, 311 cpm. (Right) IL-2-dependent release of IL-3 activity (D) Serial dilutions of rIL-2, tested on the IL-2 dependent cell line CTLL-2 (curves: 1, dilution 1:500; 2, 1:1000; 3, 1:4000; 4, 1:8000; 5, 1:16,000; 6, 1:32,000; 7, 1:128,000; 8, 1:256,000; 9, no IL-2), were added to the culture medium of C10-1 cells for 2 days of culture. (E) Supernatants were then tested for IL-3 activity on DA-1 cells.

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