REGULATION OF CYTOLYTIC CELL POPULATIONS FROM HUMAN PERIPHERAL BLOOD BY B CELL STIMULATORY FACTOR 1 (INTERLEUKIN 4)

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The generation of antigen-specific CTL is regulated by antigen and soluble growth factors. The lymphokine IL-2 is considered to play a central role in the process of CTL formation and has been shown to induce both long-term proliferation of CTL (1) and differentiation of CTL precursors to the cytolytic state (2). However, the involvement of additional soluble CTL regulatory factors has been postulated (3–8). Using a murine MLC model system, we recently demonstrated that the lymphokine B cell stimulatory factor 1 (9) (BSF-1[1] or IL-4) is, like IL-2, an extremely potent helper factor for the generation of alloreactive CTL (10). Under the conditions used in that study, IL-2 was distinguished from BSF-1 by its ability to induce a lytic population from unprimed precursors in the absence of an overt antigenic stimulus (10). Cytolytic cells generated in this manner have been observed previously in both mouse and man and have been referred to as lymphokine-activated killer, or LAK, cells (11–13). Such populations have a poorly defined target specificity and will lyse a wide array of tumor target cells (12) and, in some cases, normal lymphocytes (14).

In the experiments reported here we examined the regulation of human CTL and LAK cells by IL-2 and BSF-1. We observed that CTL generation in human MLC is augmented by BSF-1, provided it is added to cultures after an initial period of activation. However, in spite of its ability to enhance antigen-specific CTL development, BSF-1 had virtually no detectable LAK-inducing activity. In both the CTL and LAK systems, BSF-1 inhibited the development of effector cells that normally occurs in response to IL-2 when it was present together with IL-2 at the beginning of culture. These results may have important implications for immunotherapeutic strategies involving these lymphokines or cells grown in their presence.

Materials and Methods

Cells. PBMC were isolated by centrifugation over Isolymph (density = 1.077 g/cm^3; Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, NY). A portion of the cell suspensions to be used as stimulating cells was irradiated (3,000 rad) using a ^{137}Cs source. The

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[1] *Abbreviations used in this paper:* BSF-1, B cell stimulatory factor 1; LAK, lymphokine-activated killer; LU, lytic units.

tumor cell lines Daudi, A375, K562, CRL 8083, and MOLT-4 were obtained from the American Type Culture Collection, Rockville, MD. Line 7E is an EBV-transformed B cell line (15). All cell lines were grown in continuous culture.

Culture Conditions. PBMC were cultured in RPMI 1640 medium supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, and 5×10^{-5} M 2-ME. MLCs were established with 10^6 responding cells and 10^6 irradiated stimulating cells. Blasts to be used as target cells for the CTL assay were prepared by culturing freshly isolated PBMC for 3 d with 1% PHA (Gibco Laboratories, Grand Island, NY). All primary lymphoid cells were cultured in 2-ml volumes in 24-well plastic plates (No. 3524; Costar, Cambridge, MA) at 37°C in 10% CO_2.

Cell cultures to be assessed for cytolytic activity were washed twice in tissue culture medium and diluted serially in 96-well V-bottomed plates (Costar). 2×10^3 ^{51}Cr-labeled target cells were added to each well to a final volume of 0.2 ml/well. After 4 h of incubation at 37°C, the plates were centrifuged and 150 μl supernatant was removed from each well. ^{51}Cr content of supernatants was determined by using an Auto Gamma 5780 gamma counter (Packard Instrument Co., Downers Grove, IL). 1 lytic unit (LU) was defined as the fraction of the initial culture required to achieve 25 or 50% lysis of the target cell population (PHA blasts and Daudi, respectively), and was determined from dose-response curves. Spontaneous release was determined by incubating target cells in medium. Maximum release was determined by incubating target cells in 1 N HCl. Percent specific ^{51}Cr release was calculated according to the formula: 100 × [(experimental cpm) − (spontaneous cpm)]/(maximum cpm) − (spontaneous cpm)].

Lymphokines. Human rIL-2 was cloned pursuant to an agreement between Immunex Corp., Seattle, WA, and Hoffman LaRoche, Inc., Nutley, NJ, and purified to homogeneity as described in detail elsewhere (16). A human BSF-1 cDNA was cloned from a library made from RNA extracted from peripheral blood T lymphocytes (purified by E rosetting) activated for 18 h with PHA and PMA. The BSF-1 cDNA clone was isolated by hybridization with a synthetic oligonucleotide (17). rBSF-1 was produced in yeast using a plasmid that directed BSF-1 expression and secretion. Recombinant protein was purified from crude yeast supernatant by acetone precipitation and ion-exchange chromatography on Mono S (Pharmacia Fine Chemicals, Piscataway, NJ). Mono S–purified rBSF-1 was treated with *N*-glycanase (10 U, Genzyme, Boston, MA), and repurified on a ProRPC column (Pharmacia Fine Chemicals). The rBSF-1 was eluted with a linear gradient (0–100% acetonitrile in 0.1% TFA). The purified rBSF-1 migrated as a single band on SDS-PAGE with a mol mass of ~15 kD. Purified rBSF-1 had a specific activity of 10^7 U/mg as measured in a human B cell proliferation assay (18) performed as follows. Briefly, human tonsillar B cells were purified by depletion of T cells by E rosetting followed by depletion of granulocytes and monocytes by Sephadex G10 filtration. The resulting B cells (>95% pure) were assayed for their proliferative response to rBSF-1 in the presence of submitogenic concentrations of goat $F(ab')_2$ anti–human IgM, in a 72-h assay with 10^5 cells cultured in 100 μl RPMI, 10% FCS, 5×10^{-5} M 2-ME, and 50 μg/ml gentamycin.

Results

PBMC from donor VB were sensitized in MLC to irradiated cells of unrelated donor KB. IL-2 or BSF-1 was added either at initiation or after 4 d of culture to a final concentration of 10 ng/ml, a dose yielding optimal CTL generation in murine MLC (10). Cytolytic activity against VB and KB PHA blasts was assessed on day 7. The data in Fig. 1 (summarized in Table I) demonstrate that the addition to MLC of either BSF-1 or IL-2 individually resulted in increased levels of cytolytic activity directed against the specific target, KB, compared with control cultures containing no exogenous lymphokines. IL-2 enhanced CTL activity equally well when added to culture on day 0 or day 4. Interestingly, BSF-1 augmented CTL activity quite effectively when added late, but was relatively

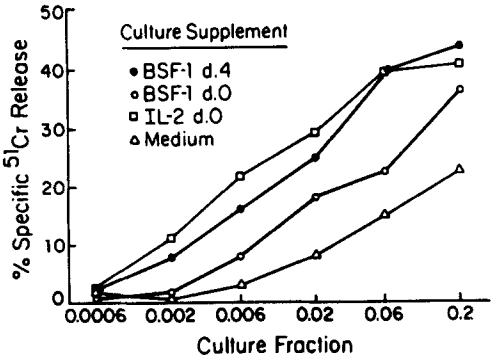

FIGURE 1. Augmentation of CTL generation in primary MLC by BSF-1 and IL-2. Cells from day 7 VB α-KB MLC, supplemented at the indicated time with 10 ng/ml IL-2 or BSF-1, were washed and mixed at the indicated fraction of the initial culture with ^{51}Cr-labeled PHA blasts from donor KB. After a 4-h incubation of effectors and target cells, supernatants were harvested and counted in a gamma counter. Spontaneous release = 186 cpm. Maximum release = 1,401 cpm.

TABLE I

Effects of BSF-1 and IL-2 on the Generation of Cytolytic Populations

Target	Culture		Culture supplement					
		Day 0:	Medium	IL-2	BSF-1	IL-2 + BSF-1	—	—
		Day 4:	—	—	—	—	IL-2	BSF-1
KB	VBαKB		~4*	100	14	18	90	50
	VBαVB		<1	<1	<1	<1	<1	<1
VB	VBαKB		<1	<1	<1	<1	<1	<1
	VBαVB		<1	<2	<1	<2	<1	<2
Daudi	VBαKB		<2	256	<2	5	56	<1
	VBαVB		<3	286	<1	7	77	<1

Cell cultures were established with 10^6 VB or KB responding cells and 10^6 irradiated (3,000 rad) stimulating cells. Human rIL-2 or rBSF-1 was added at a final concentration of 10 ng/ml at the time of culture initiation (day 0) or after 4 d. Cytolytic activity of the cultures was tested on day seven by incubating serial dilutions of the culture contents with 2×10^3 ^{51}Cr-labeled PHA blast target cells from KB or VB or with the same number of Daudi target cells. KB: spontaneous release (SR), 186 cpm; maximum release (MR), 1,401 cpm. VB: SR, 210 cpm; MR, 1,370 cpm. Daudi: SR, 158 cpm; MR, 1,541 cpm.

* Lytic units (LU) per culture. LU were calculated from the dose-response curves (see Figs. 1 and 2). Cell recoveries in MLC ranged from 147 to 254% of input responding cell number. Recoveries in syngeneic (VBαVB) cultures ranged from 87 to 152%.

The lysability of target VB was established with a control KB αVB culture supplemented with IL-2, which exhibited >600 LU of anti-VB activity.

ineffective when added early. In fact, when BSF-1 was present together with IL-2 from the beginning of culture, the lytic activity that normally develops in cultures supplemented with IL-2 alone was almost totally inhibited (Table I). The relative inefficiency of BSF-1 when added either alone or with IL-2 at day 0 was not due to a more rapid CTL response that peaked before the normal (day 7) time of assay (data not shown).

Table II shows the results of an experiment with three different blood donors. CTL were generated in MLC in which either BSF-1 or IL-2 had been added at day 3. In four of the six allogeneic cultures, the generation of lytic activity against PHA blast target cells from the stimulating cell donor was significantly enhanced (≥3-fold) by BSF-1 when compared with control cultures (GR αCL, KZ αCL, CL αGR, GR αKZ). BSF-1 showed only marginal (twofold) effects for

TABLE II
BSF-1 Enhances the Generation of Allospecific CTL

Culture	Target CL			Target GR			Target KZ		
	Medium	IL-2	BSF-1	Medium	IL-2	BSF-1	Medium	IL-2	BSF-1
KZ αCL	~5	56	58	NT*	NT	NT	<2	<2	<2
KZ αGR	NT	NT	NT	7	19	~5	<2	<2	<2
KZ αKZ	<2	<2	<2	<2	<4	<2	NT	NT	NT
GR αCL	29	91	167	<2	<2	<2	NT	NT	NT
GR αGR	<3	<4	<2	NT	NT	NT	<2	<2	<2
GR αKZ	NT	NT	NT	<2	<2	<2	<4	37	128
CL αCL	NT	NT	NT	~4	67	<2	<2	<4	<2
CL αGR	<2	<3	<2	15	77	45	NT	NT	NT
CL αKZ	<2	<4	<2	NT	NT	NT	43	139	91

Cell cultures were established with 10^6 responding cells and 10^6 stimulating cells. Lymphokines (10 ng/ml) were added at day 3. Cytolytic activity against ^{51}Cr-labeled PHA blasts was tested on day seven. Results are expressed as lytic units per culture. CL: SR, 238 cpm; MR, 1,380 cpm. GR: SR, 121 cpm; MR, 1,233 cpm. KZ: SR, 131 cpm; MR, 844 cpm.
* NT, Not tested.

one combination (CL αKZ), and no activity with another (KZ αGR). In all cases, IL-2 also enhanced CTL generation.

CTL generated in MLC supplemented with either BSF-1 or IL-2 were specific, since only minimal lytic activity was observed against target cells from the responding cell donor (Tables I and II). Furthermore, the enhancement by IL-2 or BSF-1 of CTL activity against allogeneic targets was in most cases dependent on the presence of an alloantigenic stimulus in the culture, since only weak activity developed in cultures established without allogeneic stimulating cells (Tables I and II). The single exception was the IL-2-supplemented CL αCL culture, which exhibited lytic activity against target GR. These data demonstrate that BSF-1 is a potent helper factor for the generation of antigen-specific CTL, and that the CTL generated in the presence of BSF-1 are dependent on antigen for their induction and lytic specificity.

Additional information concerning the T cell nature of the cytolytic cells generated in the presence of exogenous BSF-1 was obtained in an experiment in which MLC cells were separated on day 7 on the basis of the expression of the CD2 antigen (the cell surface receptor for SRBC) before testing in the cytotoxicity assay. ~90% of the cells from BSF-1-supplemented MLC rosetted with SRBC; this population contained the vast majority (96%) of the total lytic activity present in the unfractionated population. Thus, the antigen-specific lytic cells that arise in MLC in the presence of exogenous BSF-1 are indeed CTL.

To test the effects of IL-2 and BSF-1 on the generation of cytotoxic cells in the absence of antigen (i.e., LAK cells), the LAK-sensitive, NK cell–insensitive tumor cell line, Daudi, was used as a target (12). Data in Fig. 2 and Table I are in agreement with previous reports (12, 13) that IL-2 is a potent inducer of LAK activity. In the present experiments, strong anti-Daudi lytic activity developed when IL-2 was used to supplement either autologous VBαVB cultures or allogeneic VB α-KB MLC. In contrast, no anti-Daudi activity above that generated

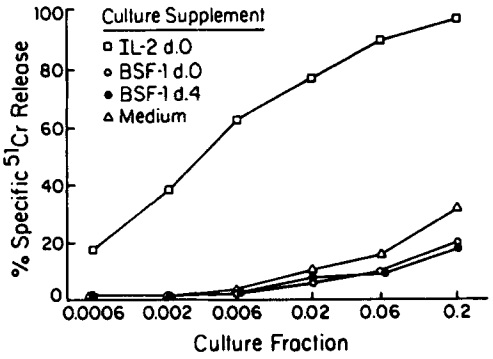

FIGURE 2. LAK activity is induced by IL-2 but not BSF-1. Cells from cultures established with 10^6 VB and 10^6 autologous, irradiated VB cells were tested on day 7 for cytolytic activity against ^{51}Cr-labeled Daudi target cells. Cultures were supplemented at 10 ng/ml with lymphokines at the indicated time. Spontaneous release = 158 cpm, maximum release = 1,541 cpm.

TABLE III

Cytolysis of Various Cultured Target Cell Lines by Lymphocytes Cultured in IL-2 or BSF-1

Target	Medium	IL-2	BSF-1
Daudi	7	63	7
A375	1	64	0
K562	20	73	23
7E	2	16	1
CRL 8083	0	12	1
MOLT-4	27	75	20

Fresh PBL were cultured for 7 d and then tested for lytic activity against the indicated cell lines. Results represent percent specific ^{51}Cr release from the indicated cell line by one-eighth of the contents of cultures supplemented with the indicated lymphokine. Spontaneous and maximum release from targets was as follows: Daudi, SR 125, MR 1350; A375, SR 1055, MR 10291; K562, SR 177, MR 3133; 7E, SR 313, MR 3404; CRL 8083, SR 164, MR 1783; MOLT 4, SR 127, MR 723.

in unsupplemented negative control cultures was detected in replicate cultures containing BSF-1. Thus, despite its potent helper activity for the generation of antigen-specific CTL, BSF-1 failed to induce LAK cells from the same donor (Table I) or in other experiments involving four additional donors. Furthermore, as observed for the generation of CTL, the development of LAK activity that normally occurs in cultures supplemented with IL-2 was inhibited when BSF-1 was present at culture initiation along with IL-2. The absence of LAK activity at day 7 in BSF-1-supplemented cultures was not due to an earlier appearance (and subsequent disappearance) of LAK cells (data not shown).

Six different continuous culture cell lines have been used as targets for killer cells generated in response to lymphokine in the absence of antigen (Table III). Whereas all six targets were lysed by cells cultured in IL-2, in no instance did lytic activity in BSF-1-supplemented cultures differ significantly from activity arising in cultures supplemented only with medium.

Table IV, Exp. 1, shows results of a dose-response experiment in which cultures supplemented with various amounts of IL-2 or BSF-1 were tested for anti-Daudi lytic activity. At concentrations of 1 ng/ml IL-2 and higher, LAK cells were readily demonstrable; however, no LAK activity was seen in replicate cultures supplemented with BSF-1, even at the highest tested concentration of 100 ng/ml.

TABLE IV

Dose-response of IL-2 and BSF-1 in CTL and LAK Generation

Lymphokine concentration	Exp. 1: LAK*		Exp. 2: CTL‡	
	IL-2	BSF-1	IL-2	BSF-1
ng/ml				
300	NT‖	NT	83	43
100	161§	<1	67	33
10	104	<1	30	63
1	33	<1	8	9
0.1	<2	<1	NT	5
0	<1		5	5

* 10^6 cells of donor GR were incubated for 7 d with the indicated dose of IL-2 or BSF-1. Lytic activity against ^{51}Cr-labeled Daudi target cells was tested on day 7.

‡ 10^6 cells of donor KZ were incubated in MLC with 10^6 irradiated (3,000 rad) cells from donor CL. Lymphokines were added to the cultures on day 4 and cytolytic activity against PHA blasts from donor CL was measured on day 7.

§ LU per culture, determined from dose-response curves as in legend to Fig. 1.

‖ NT, Not tested.

Thus, IL-2 is at least 1,000-fold more potent than BSF-1 at inducing LAK cells from human peripheral blood. This is in contrast to the effects of BSF-1 and IL-2 on antigen-specific CTL generation, where either lymphokine augmented activity at concentrations in excess of 1 ng/ml (Table IV, Exp. 2).

Discussion

The results presented here demonstrate that BSF-1 can exhibit both positive and negative regulatory effects on the in vitro generation of human antigen-specific CTL, depending on the time of its addition to culture. However, BSF-1 failed to induce any demonstrable lytic activity in the absence of an antigenic stimulus (LAK cells) and, furthermore, inhibited the IL-2-induced generation of both CTL and LAK cells when added at culture initiation.

BSF-1 enhanced CTL generation when added to MLC after a 3–4 d activation period, but was less effective when added at culture initiation. Despite its ability to amplify the generation of antigen-specific CTL, BSF-1, even when added late to cultures containing no antigenic stimulus, induced little or no LAK activity as defined by lysis of the Daudi cell line or any of five other continuous culture target cell lines. IL-2, on the other hand, showed CTL helper activity and induced LAK cells when added to culture at either time. Since these results do not reflect a difference in the kinetics of appearance of lytic cells in cultures containing exogenous IL-2 or BSF-1, they may indicate that the two lymphokines exert their effects on different cellular subsets and, perhaps, at different stages of cellular activation.

When both BSF-1 and IL-2 were present together from the beginning of culture, the generation of CTL and LAK was severely depressed compared to the activity that developed in cultures containing IL-2 but no exogenous BSF-1. It is possible that the precursors of the cytolytic or Th cells involved in the CTL

or LAK response become refractory to signals normally provided by IL-2 if they have been exposed early to BSF-1. Alternatively, BSF-1 may induce the development of a separate population of cells that suppresses CTL and LAK generation. Experiments are in progress to examine further the underlying mechanism of this phenomenon.

The cytolytic cells stimulated in MLC containing exogenous BSF-1 bear the CD2 T cell marker, require an antigenic stimulus for induction, and exhibit antigen-specific lytic activity in a short-term assay; thus, they display both cell-surface and functional properties of T cells. We have not further investigated the cellular requirements for BSF-1-induced CTL amplification. However, in murine MLC, BSF-1 stimulates CTL generation from purified $CD8^+$ CTL precursors in the absence of other T cells (10). Furthermore, BSF-1 stimulates proliferation of both activated $CD8^+$ (10, 19, 20) and $CD4^+$ (19, 20) subpopulations, as well as cloned CTL (19) and Th cells (19, 21). Thus, it is evident that BSF-1 affects CTL generation, at least in part, through its direct action on cells of the CTL lineage. However, BSF-1 may also exert some of its effects via an intermediary helper cell. In this regard, it is important to note that BSF-1-induced T cell proliferation is not affected by antibodies against IL-2 or its receptor (20, 21). Furthermore, BSF-1 failed to induce IL-2 mRNA in BSF-1-responsive T cell clones and populations (our unpublished observation).

These findings concerning the functional relationships of IL-2 and BSF-1 in terms of CTL and LAK generation have important implications for the use of these lymphokines to expand lymphoid cells for immunotherapy of cancer (22). In some in vivo model systems (23, 24), CTL or $Lyt\text{-}2^+$ populations are important in tumor elimination. Based on the in vitro results presented here, it is tempting to speculate that BSF-1, either alone or in combination with IL-2, may be useful for generating immunotherapeutic cellular populations enriched for antigen-specific CTL. It will thus be important to determine whether BSF-1 has effects on the generation of tumor-specific CTL similar to those described here in an allogeneic model system.

Summary

The effects of B cell stimulatory factor 1 (BSF-1) on the generation of human CTL and lymphokine-activated killer (LAK) cells in vitro were investigated. Both IL-2 and BSF-1 were potent helper factors for the generation of antigen-specific CTL in MLC; detection of optimal BSF-1-induced CTL activity in this system occurred when BSF-1 was added to cultures after an initial period of activation during which exogenous BSF-1 was not present. In contrast to IL-2, BSF-1 failed to induce an LAK cell population, as detected with Daudi tumor targets, in cultures that had not been allosensitized. Furthermore, when both lymphokines were added together at culture initiation, BSF-1 inhibited the IL-2-driven generation of cytolytic cells. The differential ability of BSF-1 to promote the generation of CTL but not LAK could have important implications for lymphokine-mediated immunotherapy.

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