Brief Definitive Report

INTERLEUKIN 4 (B CELL STIMULATORY FACTOR 1) CAN MEDIATE THE INDUCTION OF LYMPHOKINE-ACTIVATED KILLER CELL ACTIVITY DIRECTED AGAINST FRESH TUMOR CELLS

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In both mice and humans, the in vitro culture of normal lymphocytes in interleukin 2 (IL-2) alone is sufficient to generate cytotoxic cells that lyse an extensive spectrum of fresh natural killer (NK)-resistant tumor target cells without MHC restriction, but which do not lyse normal cells (1–3). This has been termed the lymphokine-activated killer (LAK) phenomenon. Studies from several laboratories have shown that proliferation of cytotoxic T lymphocytes and NK cells is also IL-2 dependent (4–6). Recently, the murine lymphokine B cell stimulatory factor 1 (BSF-1) has been shown to express multiple biologic activities including B cell, mast cell, and T cell stimulation (7, 8), and interleukin 4 (IL-4) has been proposed as the designation of this factor (8, 9). The cDNA clones that have been isolated from murine helper T cell lines encode a polypeptide of 120 amino acid residues that expresses these activities (8, 9). cDNA clones have also been obtained for human IL-4 (10). The existence of a polypeptide, distinct from IL-2, that also possesses a T cell growth factor activity, and the apparent strict IL-2 dependence of LAK cell proliferation and function prompted us to examine the potential LAK-inducing capacity of IL-4. We report here that murine IL-4 (native and recombinant) stimulation of murine splenocytes can result in the generation of LAK activity directed against fresh syngeneic tumor cells. The precursor cell activated by this lymphokine expresses surface asialo-GM_1. Further, we provide evidence that IL-4 can augment the generation of LAK cytolytic activity induced by IL-2.

Materials and Methods

Mice. Female C57BL/6 mice, 12–16 wk old, were obtained from The Jackson Laboratory, Bar Harbor, ME, and from the Small Animal Section, Veterinary Resources Branch, National Cancer Institute.

Tumors. MCA-102, -105, and -106 are fibrosarcomas induced by intramuscular injection of 0.1 ml of 1% 3-methylcholanthrene (MCA) into the hind limb of C57BL/6 mice by methods previously described (3). The tumors were maintained by serial passage in syngeneic hosts and were used in the third to seventh transplant generations. Single-cell suspensions were prepared from fresh tumors by short trypsinization of small tumor fragments in 0.25% trypsin in Dulbecco's PBS without calcium or magnesium (from NIH Media Unit). Released cells were pooled and washed three times in HBSS (Biofluids,

Rockville, MD). These tumor cells are resistant to lysis by NK cells and are sensitive to lysis by LAK cells (11).

Splenocyte Suspensions. Spleens were removed aseptically and crushed with the hub of a syringe in complete medium (CM). The cell suspension was passed through 100-gauge nylon mesh (Nitex; Lawshe Instrument Co., Rockville, MD), and the erythrocytes were lysed by hypotonic shock with buffered ammonium chloride solution at room temperature for 2 min. The cells were then centrifuged and washed twice in CM.

CM consisted of RPMI 1640 (Biofluids) with 10% heat-inactivated FCS (Biofluids), 0.03% fresh glutamine (NIH Media Unit), 0.1 mM nonessential amino acids (M. A. Bioproducts, Walkersville, MD), 5×10^{-5} M 2-ME (Aldrich Chemical Co., Inc., Milwaukee, WI), 100 μg/ml penicillin (NIH Media Unit), 100 μg/ml streptomycin (NIH Media Unit), 50 μg/ml gentamicin (Schering, Kenilworth, NJ), and 0.5 μg/ml Fungizone (Flow Laboratories, McLean, VA).

Lymphokines. Human rIL-2 was kindly supplied by the Cetus Corp., Emeryville, CA. The biological and biochemical activities of rIL-2 have been described (2). Purified material had a specific activity of 8×10^6 U/mg as measured in a standard bioassay using T cell line 53 (2, 3). The endotoxin level in the purified preparation was <0.1 mg per 10^6 U rIL-2 as measured by a standard limulus assay.

Native interleukin 4 (nIL-4) was purified to homogeneity from the supernatant of the Con A–induced Cl.Ly-1^+, -2^-/9 T cell line (7). rIL-4 was purified to homogeneity from the supernatant of COS-7 monkey cells transfected with a full-length cDNA clone encoding murine IL-4 (8). The specific activity of both forms was 7×10^7 U/mg in a [^{3}H]thymidine HT-2 T cell proliferation assay (7, 8).

Generation of LAK Cells. 1 ml of CM containing 4×10^6 viable C57BL/6 splenocytes was added to each well of a 24-well Costar tissue culture plate (3524; Costar, Cambridge, MA). 1 ml of CM containing varying amounts of lymphokine was also added to each well. The cultures were incubated for 4–7 d at 37°C with 5% CO_2.

Cytotoxicity Assay. A standard 4-h ^{51}Cr-release assay was used to measure cytotoxicity against tumor cells as described previously (11).

Antibody and Complement Lysis of Splenocytes. Normal splenocytes were adjusted to a concentration of 2×10^7 cells/ml of cytotoxicity medium containing a 1:50 dilution of rabbit anti–asialo-GM_1 serum (Wako Chemicals, Dallas, TX). Cytotoxicity medium consisted of RPMI 1640 with 0.3% BSA and 0.1 mM Hepes buffer. After incubation with intermittent agitation for 60 min at 4°C, cells were centrifuged and were resuspended in a half-equal volume of cytotoxicity medium with rabbit complement (Low-Tox-M; Accurate Chemical and Scientific Corp., Westbury, NY) at 1:8 dilution. Incubation was continued for an additional 60 min at 37°C with intermittent shaking. The complement treatment was repeated once again and the cells were then washed twice and were resuspended in CM.

Results and Discussion

Various concentrations of murine nIL-4, murine rIL-4, human rIL-2, or the combination of IL-4 plus IL-2 were added to C57BL/6 splenocytes and incubated for 4 d. These cells were then tested in a 4-h ^{51}Cr-release assay for the capacity to lyse the fresh, syngeneic, NK-resistant sarcoma target, MCA-102. Table I shows that 500 U of nIL-4 or 1,000 U of rIL-4/ml generated 141 ± 4 and 461 ± 94 LU of LAK activity, respectively. In addition, augmentation of cytolytic activity with the combination of IL-4 and IL-2 is evident. For example, splenocytes cultured in 500 U/ml of nIL-4 plus rIL-2 for 4 d showed 1,018 ± 335 LU, compared to 180 ± 43 and 141 ± 4 LU when activated with these concentrations of nIL-4 and rIL-2 alone.

A summary of the data from 15 separate experiments comparing LAK activity generated by native and recombinant IL-4 alone or in combination with IL-2

TABLE I
Generation of LAK Activity: Dose Titration of IL-4 and IL-2 vs. Cytolytic Activity

Source of IL-4	IL-4	rIL-2	LU* per 10^7 cells (mean ± SEM)
	U/ml		
Native	0	0	<1
	0	5	<10
	5	0	<1
	5	5	<10
	0	50	45 ± 11
	50	0	<1
	50	50	143 ± 12
	0	500	180 ± 43
	500	0	141 ± 4
	500	500	1,018 ± 335
Recombinant	0	10	63 ± 6
	10	0	<1
	10	10	102 ± 4
	0	100	1,048 ± 110
	100	0	<10
	100	100	1,045 ± 157
	0	1,000	648 ± 69
	1,000	0	461 ± 94
	1,000	1,000	2,004 ± 335

C57BL/6 mouse splenocytes were cultured at 2×10^6 cells/ml in 2 ml of complete medium containing varying amounts of lymphokine. After 4 d the cells were tested for cytolytic activity against ^{51}Cr-labeled, fresh MCA-induced sarcoma cells (MCA-102) in a 4-h assay.

* 1 LU is defined as the number of effector cells mediating 30% specific lysis of 10^4 target cells, and is determined from the dose-response curve. LU presented as mean ± SEM of triplicate wells.

against three distinct MCA-sarcoma targets (102, 105, 106) is shown in Table II. Interexperiment variability in the generation of LAK activity by IL-2 or IL-4 is evident, since in most experiments suboptimal concentrations of lymphokines were tested, and we used freshly-resected tumor cells rather than cultured lines as targets. The recoveries of splenocytes from 4- or 5-d cultures (as a percentage of the initial number of cells plated) varied slightly between experiments, and were ~50% for IL-2 and 30% for IL-4; those for IL-4 plus IL-2 were equivalent to IL-2 alone.

We reported previously that the murine precursor cell that gives rise to LAK cells after rIL-2 stimulation expresses surface asialo-GM_1 (11). Thus we analyzed whether or not the precursor activated by IL-4 similarly expressed surface asialo-GM_1 by pretreating normal C57BL/6 splenocytes with specific heteroantiserum plus complement before lymphokine activation. Fig. 1 shows that depletion of asialo-GM_1–positive cells resulted in a near complete elimination of both the IL-

TABLE II

IL-4 Generates LAK Activity and Augments This Activity in Combination with IL-2

Exp.*	Source of IL-4	Tumor target (MCA)	LU per 10^7 cells (mean ± SEM) with:		
			IL-4	IL-2	IL-4 + IL-2
1	Native	102	141 ± 4	180 ± 43	1,018 ± 335
2a		102	133 ± 34	305 ± 80	1,060 ± 342
b		102	81 ± 38	276 ± 84	1,469 ± 315
3		102	63 ± 6	95 ± 21	318 ± 73
4a		102	114 ± 14	90 ± 5	105 ± 5
b		105	227 ± 18	79 ± 9	973 ± 140
5a		102	219 ± 27	204 ± 12	1,446 ± 117
b		105	50 ± 7	58 ± 11	620 ± 109
6a		102	130 ± 13	275 ± 44	277 ± 67
b		105	1,007 ± 284	490 ± 38	1,312 ± 280
7		105	<10	<10	71 ± 9
8	Recombinant	102	461 ± 94	648 ± 69	2,004 ± 335
9		102	76 ± 21	143 ± 35	655 ± 298
10a		102	<10	38 ± 6	179 ± 16
b		105	57 ± 8	<10	616 ± 127
11		105	44 ± 11	<10	147 ± 14
12		105	<10	50 ± 20	78 ± 32
13a		102	62 ± 4	148 ± 18	226 ± 29
b		105	66 ± 8	<10	192 ± 73
14		106	42 ± 7	77 ± 27	124 ± 22
15a		102	171 ± 12	231 ± 33	1,372 ± 77
b		105	340 ± 87	<10	1,778 ± 224

* 5-d and 7-d splenocyte cultures were used in experiments 2a, 5, 6, 15, a and b, and in experiment 2b, respectively; 4-d splenocyte cultures were used in all other experiments. Cultures contained 500 U/ml of each lymphokine per milliliter of CM, except for experiment 8, in which the concentration was 1,000 U, and in experiments 5, 6, and 15, a and b, in which 200 U rIL-2 and 1,000 U IL-4 were used.

4 and the rIL-2-induced LAK activity. Furthermore, preliminary experiments (Mulé, Smith, and Rosenberg, unpublished results) indicate that specific depletion of mature T cells has little detectable effect on LAK generation by IL-4. IL-4 generated killer cells do not appear to lyse normal cells. For example, in an experiment in which IL-4-generated killer cells showed 137 LU of activity against the MCA-102 sarcoma target, <1 LU of activity was noted against syngeneic fresh peripheral blood lymphocytes, bone marrow, kidney, lung, and LPS-stimulated splenic blasts.

Taken together, these results demonstrate that the lymphokine IL-4 is capable of inducing cells with LAK activity that lyse fresh tumor cell targets in vitro from an asialo-GM_1–positive precursor. IL-4 and IL-2 precursor-effector relationships, as well as the effector phenotype of IL-4-activated killer cells, however, remain to be determined. Preliminary experiments (Mulé et al., unpublished results) indicate that, unlike IL-2, IL-4 has little if any capacity to generate LAK activity from mouse peripheral blood lymphocytes, whereas both lymphokines induce this activity from splenocytes. Whether or not purified human IL-4 similarly generates LAK activity from human lymphocytes remains to be determined.

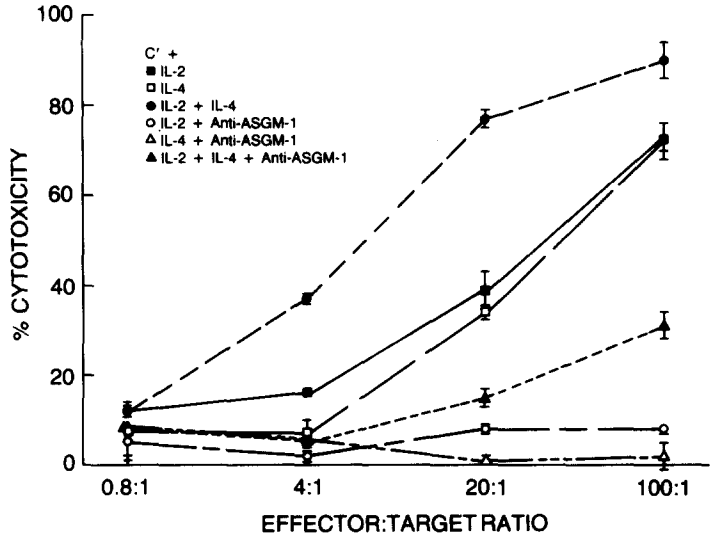

FIGURE 1. IL-4-activated killer cells arise from asialo-GM_1–positive precursors. Splenocytes were activated in vitro for 4 d in 500 U/ml of lymphokine of CM and were tested in vitro against fresh MCA-102 target cells. Native IL-4 was used in this experiment. Cytolytic activity was not detected from splenocyte cultures after elimination of asialo-GM_1–positive cells by heteroantiserum and complement. Symbols and bars represent means and standard errors of measurements of triplicate wells.

IL-4 has been shown to have diverse biologic activities that include: (*a*) costimulation with anti-μ antibody to induce proliferation of resting B cells (8, 9), (*b*) Ia induction on B cells (12, 13), (*c*) selective enhancement of IgG1 (14) and IgE (15) production by mitogen-activated B cells, (*d*) a mast cell growth factor activity that synergizes with IL-3, and a T cell growth factor activity (7, 8). The results reported here expand the IL-4 activities to include the induction of LAK activity from normal resting splenocytes as well as the augmentation of this activity in combination with IL-2.

Summary

Interleukin 4 (IL-4) expresses multiple biologic activities, including B cell, mast cell, and T cell stimulation. We showed that the incubation of resting splenocytes from C57BL/6 mice solely in purified native or recombinant mouse IL-4 results in the generation of lymphokine-activated killer (LAK) activity directed against fresh, syngeneic sarcoma cells. The precursor activated by IL-4 expresses surface asialo-GM_1. In addition, IL-4 is capable of amplifying the splenic LAK activity induced by recombinant IL-2. The generation, by IL-4, of killer cells with broad antitumor reactivity raises the possibility of using IL-4 alone or in combination with IL-2 in the immunotherapy of cancer in animal models.

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