Brief Definitive Report

LYMPHOKINE-ACTIVATED KILLER CELL PHENOMENON

III. Evidence that IL-2 Is Sufficient for Direct Activation of Peripheral Blood Lymphocytes into Lymphokine-activated Killer Cells

By E. A. GRIMM,* R. J. ROBB,§ J. A. ROTH,* L. M. NECKERS,‡ L. B. LACHMAN,‖ D. J. WILSON,* and S. A. ROSENBERG*

*From the *Surgery Branch and the ‡Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland 20205; the §Central Research and Development Department, E. I. du Pont de Nemours & Company, Glenolden Laboratory, Glenoldon, Pennsylvania 19036; and the ‖Department of Cell Biology and Interferon Research, M. D. Anderson Hospital, Houston, Texas 77030*

In both murine and human systems, incubation of fresh lymphocytes alone in lectin-free lymphokine preparations rich in interleukin 2 (IL-2) results in the development of lytic activity against fresh autologous and allogeneic solid tumor cells (1–4). The human lymphokine-activated killer cells (LAK) represent a cytotoxic system distinct from conventional cytotoxic T lymphoid cells and natural killer (NK) cells based on a variety of characteristics including kinetics of activation, target cell specificity, the stimulus responsible for activation, and phenotype of the precursor and effector cells (2–5).

While we have consistently observed LAK activation only after incubation of PBL with cytokine preparations containing IL-2, it was possible that due to the heterogeneous nature of our standard lectin-free IL-2 preparations, other lymphokine(s) or monokine(s) could be responsible or could act synergistically with IL-2 to activate LAK. Therefore, this study was undertaken to define the cytokine(s) responsible for the direct activation of nonlytic PBL into LAK cells. The results show that the LAK stimulus adsorbs significantly to IL-2-dependent murine cell lines, as does all detectable IL-2 activity. IL-2 preparations from both the Jurkat leukemia line (6, 7), and from the MLA-144 line (8) purified to apparent molecular homogeneity were both highly active in the generation of LAK. Anti-Tac antibody that recognizes the IL-2 receptor (9, 10) was observed to block LAK activation. Purified preparations of IL-1, migration inhibition factor (MIF), interferon (IFN), and IFN-γ produced by recombinant DNA technology were all negative for activation of LAK. Thus, we conclude that IL-2 alone directly activates LAK precursors into cytolytic effector cells that are efficient in lysing single-cell suspensions of noncultured solid tumor cells in a 4-h chromium-release assay.

Materials and Methods

IL-2 Preparations and Purification. Lymphokine supernatants rich in IL-2 activity were prepared by stimulation of human PBL with phytohemagglutinin (PHA) as described in detail previously (11, 12). The crude IL-2 preparation was then partially purified accord-

ing to the procedure of Meir and Gallo (13), and was used as the starting material for the IL-2 adsorption studies reported in Fig. 1.

IL-2 from the Jurkat human leukemia line (14) was obtained after stimulation with PMA and PHA as described (15). Jurkat IL-2 was purified using adsorption to an anti-IL-2 affinity column using a murine monoclonal antibody (16). The IL-2 that was eluted from the column appeared homogeneous by two-dimensional gel electrophoresis using isoelectric focusing (IEF) in the first dimension, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16).

IL-2 purified from the cultured supernatant of the Gibbon ape MLA-144 was the generous gift of Dr. Louis Henderson and Dr. Harvey Rabin, Frederick Cancer Research Center, Frederick, MD. MLA IL-2 was purified to apparent molecular homogeneity by trimethylsilane-controlled pore glass adsorption, followed by reverse-phase high performance liquid chromatography as described by Henderson et al. (17). All lymphokine preparations were tested for IL-2 content by their ability to stimulate [^{3}H]thymidine uptake in a 48-h assay using human IL-2-dependent cell lines as previously described (11, 12).

Adsorption of Human IL-2 by IL-2-dependent Lymphocyte Lines. IL-2-addicted murine cells (line 53) were harvested, washed free of culture medium, and incubated at 37°C for 2 h in RPMI 1640 medium (5×10^6 cells/ml) containing 10% human AB serum (CM). These cells were then washed twice in serum-free RPMI 1640 and divided into aliquots of 5×10^7 each. 1 ml of a dilution of human IL-2 in RPMI 1640 was added to each aliquot of line 53 cells, and adsorption performed at room temperature for 2 h with occasional mixing. This first adsorption was terminated by centrifugation of the cells, after which the supernatant was aspirated and added to a second 5×10^7 cell pellet for an additional adsorption step.

Preparation and Purification of Other Cytokines. Human IL-1 was prepared from Ficoll-Hypaque–purified PBL by culturing for 48 h at 3×10^6/ml in minimal essential medium containing 5% pooled normal human serum and 10 μg/ml *Escherichia coli* lipopolysaccharide (LPS) (Difco Laboratories Inc., Detroit, MI). IL-1 was purified by hollow fiber diafiltration and ultrafiltration, followed by sucrose gradient IEF, and activity was determined in the mouse thymocyte [^{3}H]thymidine incorporation assay as previously described (18). The IEF-purified material expressed biological activity at 0.1% (vol/vol) and contained no IL-2 activity.

Two preparations of leukocyte MIF devoid of IL-2 were the generous gift of Dr. H. Verheul, Organon, OSS, Holland (19) and represent the supernatant of human lymphoblastoid cell line cultures CP 82004 and 82DB02. The specific MIF activity was 36 and 17 U/mg, respectively. IFN-γ produced by recombinant DNA technology was generously provided by Genentech, Inc., San Francisco, CA. Purified alpha and gamma IFN produced from PBL was purchased from Interferon Sciences, Inc., New Brunswick, NJ.

LAK Activation and Testing for Cytotoxicity. PBL at 1×10^6/ml were cultured in CM containing 10% human AB serum (10 ml maximum) for 4–6 d at 37°C with various dilutions of IL-2 or other factors in Costar 3050 flasks (Costar, Cambridge, MA). The cells were then harvested, washed twice in CM, counted, and used as effector cells for lysis of NK-resistant, cryopreserved, uncultured sarcoma cells. These cells were composed of a minimum of 93% tumor cells as judged by cytological analysis (3). Lysis was tested in a 4-h ^{51}Cr-release assay at a variety of effector/target ratios and data is reported as either percent specific lysis (2) or in terms of lytic units (LU), defined as the number of effector cells required to cause a 33.3% lysis of 5×10^3 tumor cells.

Results and Discussion

LAK Activation Signal(s) Copurifies with IL-2 During Protein Purification Procedures. Since LAK stimulation always occurred when IL-2-rich lymphokine supernatants were used, our initial approach to dissection of the signal(s) responsible for LAK activation was to apply the standard IL-2 purification procedure

and follow in parallel the separation of IL-2 and LAK activation factor(s). We consistently observed LAK activation using IL-2-enriched fractions from ammonium sulfate precipitation and DEAE anion exchange chromatography. Furthermore, we previously reported (20) that these two activities copurified during ACA 54 Ultragel separation, confirming that IL-2 and the LAK stimulatory signal(s) share similar physical properties of molecular size (used in ACA Ultragel separation) and ionic/hydrophobic characteristics (used in DEAE anion exchange chromatography).

LAK Stimulatory Signal Adsorbs to IL-2-dependent Cells. Fig. 1 displays the results from one of three adsorption experiments in which the lectin-free, partially purified IL-2 and the LAK stimulatory signal(s) were consistently adsorbed by IL-2-dependent cell lines. Adsorption was effective in removing all IL-2 activity and in causing a 4–16-fold decrease in the generation of LAK activity measured in LU.

LAK Activation is Inhibited by Monoclonal Anti–IL-2-receptor Antibodies. The monoclonal antibody, anti-Tac, has been reported to recognize the IL-2 receptor and block IL-2 binding (9, 10). When dilutions of anti-Tac ascites (generously provided by Dr. Thomas Waldmann, Metabolism Branch, National Cancer Institute) were added to the initiation of LAK culture on day 0 at the same time as the partially purified lectin-free IL-2, a dose-dependent inhibition of LAK activation was observed (Table I). In contrast, addition of anti-Tac to the 4-h cytotoxicity test had no effect, even at the 1:500 dilution. Therefore, the anti-Tac antibody appeared to inhibit the activation phase of LAK, implying that the binding of IL-2 to its receptors is an obligatory step in LAK cell activation.

Purified IL-2 Activates LAK. The preceding data provides very strong yet circumstantial evidence that LAK cells are stimulated directly by IL-2. To test this in a conclusive manner, two preparations of IL-2 purified to molecular homogeneity were used. Both the Jurkat-derived (16) and MLA-144-derived IL-2 were highly stimulatory for LAK over a very broad concentration range (Table II).

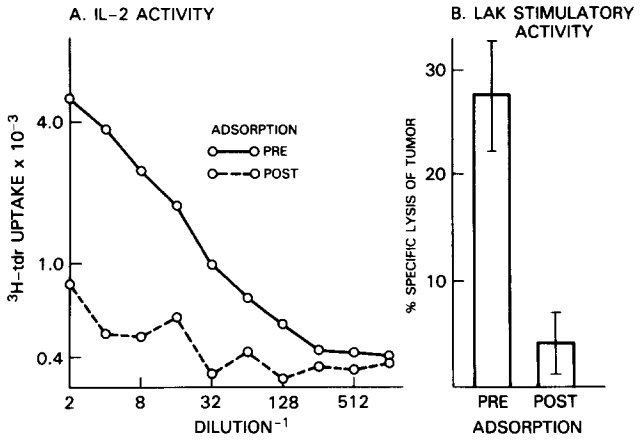

FIGURE 1. Adsorption of IL-2 and LAK stimulatory activities by IL-2-dependent cell lines. IL-2 was adsorbed as described in Materials and Methods. Pre- and post-adsorption samples were tested for IL-2 content (*A*) and for LAK stimulation (*B*). ^{3}H-tdr, [^{3}H]thymidine.

Though we have confirmed that highly purified IL-2 directly activates LAK cells, IL-2 may not be exclusive in this property. Therefore, we have acquired and tested a number of other human cytokines that are able to stimulate mononuclear cell functions. IL-1, MIF, and a variety of IFN preparations tested at several log-fold dilutions surrounding the optimal dose (optimal dose determined independently for each cytokine preparation; data not shown) were unable to generate LAK activity when incubated with fresh PBM. Atlhough IFN are known to augment suboptimal NK activity, as well as being produced in response to IL-2, we found them unable to directly convert noncytolytic LAK precursors into cytolytic cells active against NK-resistant tumors.

IL-2 was reported originally not to be stimulatory for nonactivated lymphocytes because in a 2-d [^{3}H]thymidine-uptake assay, the stimulation is negligible relative to IL-2-dependent cells. Lectin-free IL-2 preparations, however, do cause detectable [^{3}H]thymidine uptake above the level in control cultures and can be used to initiate long-term growth from these nonactivated populations (11, 12). Direct activation of PBL into LAK cells by purified IL-2 suggests that receptors for IL-2 are expressed on some PBL. Additional evidence for the existence of IL-2 receptors on PBL is suggested by the radiolabeled IL-2-binding studies of Robb et al. (7) in which a low number (~210/cell) of receptors was found on resting PBL.

The exact nature of the LAK precursor is still not understood. It was possible that the LAK precursors were the multiclonal memory T cells that may be circulating in the PBL, since it is known that secondary cytotoxic T lymphocytes (CTL) can be generated from memory T cells directly by IL-2 (5). However, the LAK precursors did not express the serologic phenotype of memory CTL, when tested in parallel with allospecific memory CTL generated in vitro (5). Theseresults imply that two subpopulations of PBL may respond directly to IL-2: the memory CTL, which express a T cell phenotype, and the LAK precursors, which do not.

Summary

Purified interleukin 2 (IL-2) was found to be sufficient for direct activation of peripheral blood lymphocytes into lymphokine-activated killer (LAK) cells. The LAK activation factor was directly and consistently associated with IL-2 activity

TABLE I

*Inhibition of LAK Development by Anti-Tac**

Anti-Tac dilution	LAG generated (LU/10^6 cells)‡	Percent inhibition
0	111	—
1:16,000	71	36
1:4,000	25	77
1:2,000	17	85
1:1,000	13	89
1:500	5	96

* Anti-Tac was added at the initiation of cultures of PBL with IL-2. After 5 d of incubation, the cells were harvested, counted, and tested for lysis of fresh sarcoma tumor target cells at a variety of effector/target ratios. The cell recoveries in the cultures containing anti-Tac were from 50 to 88% of the control culture.

‡ LU were calculated by extrapolating the number of effector cells required to cause a 33.3% lysis of 5×10^3 tumor cells.

TABLE II

Activation of LAK by Lymphokines

Preparation	Source	Reference*	Concentrations tested‡	LAK (LU/10^6 cells)§
IL-2				
DEAE fraction (lectin-free)	PBL (0.14 U/mg)	This paper	0.04–0.4 U/ml	14.3
ACA 54 Ultragel fraction	PBL (0.7 U/mg)	Fig. 1, this paper	0.25U/ml	7.4
MLA-144 culture, supernatant purified (170,000 U/mg)	—	H. Rabin, Frederick Cancer Research Center	0.25–1.0 U/ml	≥50
Jurkat line, supernatant purified (320,000 U/mg)	—	R. Robb, E. I. du Pont de Nemours & Co.	0.2–3.6 U/ml	≥50
Other cytokines				
IL-1, purified to molecular homogeneity (10% optimal)	PBL	L. Lachman, Immunex	3–25%	0
MIF, free of IL-2	Lymphoblastoid cell lines	H. Verhuel, Organon International Bv.	25–500 μg/ml	0
Interferon α	PBL, ultrapure (1×10^8 IU/mg)	Interferon Sciences, Inc.	10–10,000 U/ml	0
Interferon γ	PBL (≥1×10^6 IU/mg)	Interferon Sciences, Inc.	10–500 U/ml	0
Interferon γ from recombinant DNA	—	Genentech, Inc.	0.1–10,000 U/ml	0

* See Materials and Methods for the complete reference for each cytokine.

‡ Each cytokine was tested at the maximal concentration available, and at least four dilutions lower, covering the optimal range of its inherent activity (data not shown).

§ (See Materials and Methods) are reported for the highest LAK activity observed toward the NK-resistant sarcoma tumor target cell.

using classic protein purification techniques, adsorption to IL-2-dependent cell lines, and inhibition with anti-Tac antibody. As yet, no other cytokines have been found that perform the same role.

Received for publication 19 May 1983 and in revised form 2 August 1983.

References

1. Yron, I., T. A. Wood, P. J. Spiess, and S. A. Rosenberg. 1980. In vitro growth of murine T cells. V. The isolation and growth of lymphoid cells infiltrating syngeneic solid tumors. *J. Immunol.* 125:238.
2. Lotze, M. T., E. A. Grimm, A. Mazumder, J. L. Strausser, and S. A. Rosenberg. In vitro growth of cytotoxic human lymphocytes. 1981. IV. Lysis of fresh and cultured autologous tumor by lymphocytes cultured in T cell growth factor. *Cancer Res.* 41:4420.
3. Grimm, E. A., A. Mazumder, H. Z. Zhang, and S. A. Rosenberg. 1982. Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J. Exp. Med.* 155:1823.
4. Grimm, E. A., E. Gorelik, M. M. Rosenstein, and S. A. Rosenberg. 1982. The lymphokine-activated killer cell phenomenon: in vitro and in vivo studies. *In* The Proceedings of the Third International Lymphokine Congress. S. Cohen and J. Oppenheim, editors. Academic Press, Inc., New York. p. 739.
5. Grimm, E. A., D. J. Wilson, and S. A. Rosenberg. 1983. Lymphokine-activated killer

cell phenomenon. II. The precursor phenotype is serologically distinct from peripheral T lymphocytes, memory CTL, and NK cells. *J. Exp. Med.* 157:884.

6. Gillis, S., and J. Watson. 1981. Biochemical and biological characterization of lymphocyte regulatory molecules. V. Identification of an interleukin 2-producing human leukemia T cell line. *J. Exp. Med.* 152:1709.
7. Robb, R. J., A. Munck, and K. A. Smith. 1981. T cell growth factor receptors. Quantitation, specificity, and biological relevance. *J. Exp. Med.* 154:1455.
8. Rabin, H., R. F. Hopkins, F. W. Ruscetti, R. H. Neubauer, R. L. Brown, and T. G. Kawakami. Spontaneous release of a factor with properties of T cell growth factor from a continuous line of primate tumor T cells. *J. Immunol.* 127:1852.
9. Uchiyama, T., S. Broder, and T. A. Waldman. 1981. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. *J. Immunol.* 126:1393.
10. Leonard, W. J., J. M. Depper, T. Uchiyama, K. A. Smith, T. A. Waldman, and W. C. Greene. 1982. A monoclonal antibody that appears to recognize the receptor for human T cell growth factor and partial characterization of the receptor. *Nature (Lond.).* 300:267.
11. Grimm, E. A., and S. A. Rosenberg. 1982. Production and properties of human IL-2. *In* Isolation Characterization and Utilization of T Lymphocyte Clones. G. Fathman and F. Fitch, editors. Academic Press, Inc., New York. 57–81.
12. Lotze, M. T., and Rosenberg, S. A. 1981. In vitro growth of cytotoxic human lymphocytes. III. The preparation of lectin-free T cell growth (TCGF) and an analysis of its activity. *J. Immunol.* 126:2215.
13. Meir, J. W., and R. C. Gallo. 1982. The purification and properties of human T cell growth factor. *J. Immunol.* 128:1122.
14. Kaplan, J., J. Tilton, and W. D. Peterson, Jr. 1976. Identification of T-cell lymphoma tumor antigens on human T-cell lines. *Am. J. Hematol.* 1:219.
15. Robb, R. J. 1982. Human T-cell growth factor: purification, biochemical characterization, and interaction with a cellular receptor. *Immunobiology.* 161:21.
16. Robb, R. J., R. M. Kutny, and V. Chowdhry. Purification and partial sequence analysis of human T-cell growth factor. *Proc. Natl. Acad. Sci. USA.* In press.
17. Henderson, L. E., J. F. Hewetson, R. F. Hopkins, R. C. Sowder, R. H. Neubauer, and H. Rabin. 1983. A rapid large scale purification procedure for gibbon interleukin 2. *J. Immunol.* In press.
18. Lachman, L. B., S. O. Page, R. S. Metzgar. 1980. Purification of human interleukin 1. *J. Supramol. Struct.* 13:457.
19. H. A. M. Verheul, F. C. den Hollander, D. M. Dijkhuizen, J. W. L. Gijzen, J. G. M. Kessels, and A. Schuurs. 1982. Production and Characterization of lymphoid cell-line (LCL-LK) preparations. *In* Human Lymphokines. A. Khan and N. O'Hill, editors. Academic Press, Inc., New York. 343–352.
20. Grimm, E. A., and S. A. Rosenberg. The human lymphokine-activated killer cell phenomenon. *In* The Lymphokines. E. Pick and M. Lundy, editors. Academic Press, Inc., New York. In press.