Antiviral Effect of Lymphokine-Activated Killer Cells: Chemotaxis and Homing to Sites of Virus Infection

ROBERT J. NATUK,[†] JACK F. BUKOWSKI, JEFFERY O. BRUBAKER, AND RAYMOND M. WELSH*

Department of Pathology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

Received 9 June 1989/Accepted 25 July 1989

Lymphokine-activated killer (LAK) cells generated from C57BL/6 mouse spleen cells cultured with interleukin-2 are effective prophylactically against virus infection when inoculated at the site of virus injection. To predict the therapeutic efficacy of LAK cells, we determined whether LAK cells would home to sites of virus infection. In vitro, LAK cells responded chemotactically to cell-free peritoneal exudate fluids collected from virus-infected mice and to preparations of purified beta interferon. In vivo, radiolabeled LAK cells injected intravenously accumulated in the peritoneal cavities of intraperitoneally infected mice in amounts three to eight times greater than in uninfected mice. This ability to respond to chemotactic agents and migrate into sites of virus infection may make LAK cells useful as antiviral therapeutic agents.

Lymphokine-activated killer (LAK) cells are cytotoxic effector cells generated by culturing spleen or peripheral blood leukocytes in high concentrations of interleukin-2 (IL-2) (3-5, 7, 12, 16, 19). The nature of LAK cells has been controversial, but many researchers agree that most of the cytotoxicity in 5-day LAK cell cultures is mediated by activated natural killer (NK) cells (3, 5, 7, 12, 16). A small but significant proportion of the killing is mediated by T-like cells that express CD3 and/or CD8 antigens. These T-like cells appear to lyse targets nonspecifically but with a target range hierarchy of sensitivity different from that of NK cells (3-7, 12, 19).

LAK cells, particularly in combination with IL-2 infusions, have been shown to mediate antitumor effects in humans and murine animal models (9, 15). This property is consistent with that of NK cells, which have been shown to inhibit tumor implantation, growth, and metastases (18). NK cells have also been shown to provide resistance to certain virus infections of mice, such as murine cytomegalovirus (MCMV) (2). Because LAK cells resemble activated NK cells, we tested their ability to protect mice against virus infection. The results indicated that as few as 2×10^5 LAK cells inoculated intraperitoneally (i.p.) into suckling mice protected these mice from i.p. infection with MCMV but not from infection with NK cell-resistant lymphocytic choriomeningitis virus (LCMV) (3).

NK cells accumulate to high levels at sites of virus infection, such as the peritoneal cavity (10). These NK cells are activated and respond chemotactically to extracts from virus-infected tissue, to purified beta interferon (IFN- β), and to recombinant IL-2 (10, 11). Intraperitoneal injections of IFN- β and/or IL-2 stimulate the accumulation of NK cells in the peritoneal cavity (R. J. Natuk and R. M. Welsh, unpublished data). Virus-induced blast cytotoxic T lymphocytes respond similarly to the same chemotactic agents (11). Presumably, the accumulation of activated cytotoxic effector cells at sites of virus infection is essential for their antiviral functions.

The fact that LAK cells are active prophylactically against infections with NK cell-sensitive viruses suggests that they might also be useful in therapeutic regimens. In planning

strategies for antiviral therapy using LAK cells, we questioned whether these culture-generated cells could respond to virus-induced chemotactic factors and accumulate in virus-infected tissue after inoculation in vivo. Here we report that LAK cells do respond chemotactically and do accumulate at sites of virus infection.

LAK cells were generated by culturing spleen cells from 6- to 12-week-old C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine) for 5 days in RPMI 1640 medium supplemented with antibiotics, glutamine, 10% heat-inactivated (56°C, 30 min) fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids (M. A. Bioproducts, Walkersville, Md.), and 1,000 U of highly purified human recombinant IL-2 from *Escherichia coli* per ml, kindly provided by Cetus Corp., Emeryville, Calif. (14, 17). Leukocytes were cultured at starting concentrations of 3×10^6 /ml. These culture-generated LAK cells, which were highly cytotoxic against a wide variety of target cells, were tested in chemotaxis assays using Boyden blind-well chemotactic chambers (Nuclepore Corp., Pleasanton, Calif.) with the top and bottom chambers separated by two 5- μ m-pore-size polycarbonate Nuclepore filters as previously described (10, 11). Cells that migrated into the bottom chambers were counted to determine the degree of chemotactic response. Representative data in Table 1 show the responses of LAK cells to a variety of agents. LAK cells migrated well in response to purified IFN- β (Lee Biomolecular, La Jolla, Calif.) and to peritoneal exudate fluid from mice infected i.p. with mouse hepatitis virus (MHV) strain A59 or vaccinia virus (VV) strain IHDJ ($P < 0.01$). The IFN- β titers in similarly produced MHV-induced peritoneal exudates ranged from 160 to 2,560 U/ml. Neither peritoneal fluid from uninfected control mice (Table 1, experiment 2) nor a commercially prepared mock IFN- β control used at the same concentration as IFN- β attracted LAK cells (Table 1, experiment 3). IFN- β appeared to be primarily chemotactic and not chemokinetic during these assays (7 h), since addition of IFN- β to the top chamber did not enhance migration into the bottom chamber (Table 1, experiments 3 and 4). Migration of LAK cells toward IFN- β was IFN- β dose dependent, and significant migration was observed at 1,000 U of IFN- β per ml, which is within normal physiological concentrations (Table 1, experiment 5). IL-2 was only marginally chemotactic for LAK cells (Table 1,

* Corresponding author.

[†] Present address: Wyeth Laboratories, Philadelphia, PA 19101.

TABLE 1. Chemotactic response of LAK cells in vitro^a

Expt no. and chemotactic agent (amt [U])	Mean $\pm$ SD no. of cells that migrated
1	
RPMI	75 $\pm$ 7.4
MHV-induced peritoneal fluid	260 $\pm$ 38
VV-induced peritoneal fluid	400 $\pm$ 53
IFN- β (5×10^3)	670 $\pm$ 36
Human recombinant IL-2 (180)	110 $\pm$ 14
2	
RPMI	12 $\pm$ 2.8
Control peritoneal fluid	18 $\pm$ 3.0
MHV-induced peritoneal fluid	67 $\pm$ 25
3	
RPMI	76 $\pm$ 8.9
IFN- β (5×10^3)	210 $\pm$ 29
IFN- β (5×10^3 ; top)	50 $\pm$ 3.2
Mock IFN- β	76 $\pm$ 4.0
Mock IFN- β (top)	64 $\pm$ 3.2
4	
RPMI	11 $\pm$ 3.2
IFN- β (5×10^3)	220 $\pm$ 1.6
IFN- β (5×10^3 ; top)	4.8 $\pm$ 5.7
Human recombinant IL-2 (100)	17 $\pm$ 11
5	
Minimum essential medium	44 $\pm$ 17
IFN- β (10^4)	166 $\pm$ 15
IFN- β (10^3)	77 $\pm$ 9.9
IFN- β (10^2)	55 $\pm$ 12

^a LAK cells were prepared from normal 6- to 12-week-old female C57BL/6J mice and cultured for 5 days. Cells were harvested and washed with serum-free RPMI medium and adjusted to 5×10^6 cells per ml in serum-free RPMI medium. Cells in a 0.2-ml volume were loaded into the top chamber of a Boyden blind-well chemotactic apparatus. The bottom chamber contained serum-free RPMI, mouse IFN- β , a mock IFN- β preparation, human recombinant IL-2, or cell-free peritoneal washout fluids collected from uninfected mice or mice infected i.p. with either MHV or VV in 4.0 ml of serum-free RPMI medium. The two chambers were separated by two 13-mm-diameter polycarbonate membrane filters, each with a pore size of 5.0 μ m. In some experiments, presumptive chemotactic agents were placed in the top chamber with the LAK cells to measure chemokinetic activity. After 7 h of incubation in a 37°C humidified CO₂ chamber, the nonmigrated cells present in the top chamber were removed, and the top chamber was washed twice. In experiments 1 to 4, the filters were removed and the medium from the bottom chamber was collected and mixed with 0.2 ml of RPMI medium containing 10% fetal bovine serum, and 0.1-ml samples were used for cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, Pa.) preparation (600 rpm, 4 min). The cytocentrifuge pellets were stained with Wright-Giemsa and examined microscopically. The average number in 10 microscopic fields (magnification, $\times 40$) was determined. In experiment 5, the migrated cells were diluted, counted directly in a hemacytometer, and expressed as the number of cells per hemacytometer field. All data are from replicas of at least three separate Boydon chambers.

experiments 1 and 3), but this concentration of IL-2 was highly chemotactic for freshly harvested activated NK cells and cytotoxic T lymphocytes from virus-infected mice (11). Our data presented here and elsewhere (10, 11) therefore indicate that activated NK cells, cytotoxic T lymphocytes, and LAK cells all respond well to exudates from virus-infected tissue, but LAK cells respond relatively better to purified IFN- β and more poorly to recombinant IL-2. Perhaps the discrepancy in the chemotactic responses to IFN- β and IL-2 relates to the fact that LAK cells are exposed to very high concentrations of IL-2 in vitro, and NK cells and cytotoxic T lymphocytes are exposed to very high concentrations of IFN- β in vivo. Saturation or down regulation of

INOCULATION

FIG. 1. C57BL/6J mice (6 to 12 weeks old; three to six per group) were injected intravenously via the retro-orbital sinus with 10^7 LAK cells at 1, 2, or 3 days after i.p. or footpad (f.p.) infection with viruses. LAK cells were generated by incubation of normal C57BL/6J spleen cells in human recombinant IL-2 for 5 days. LAK cells were labeled with 125 I-labeled iododeoxyuridine at 1.0 μ Ci/ 3×10^6 cells per ml overnight, beginning on day 4 of incubation. Labeled LAK cells were collected, washed, adjusted to 10^8 cells per ml, and injected intravenously into mice infected with the following viruses: MCMV, Smith strain (2×10^5 PFU); LCMV, Armstrong strain (8×10^4 PFU); MHV strain A-59 (2×10^5 PFU); or VV strain IHDJ (1.7×10^7 PFU). Control mice were either injected with RPMI medium (B) or left unmanipulated (A and C). At 2 (A and B) or 6 (C) h postinjection of labeled LAK cells, the animals were anesthetized with ether and exsanguinated, and the peritoneal cavities were lavaged with 4.0 ml of medium. The total amount of radioactivity per cell pellet of the peritoneal exudate was determined. The data are expressed as the number of cell-equivalent counts per minute (10^4). Standard deviations (error bars) and *P* values were determined for each experiment. Cell migration into the peritoneal cavity for each group inoculated i.p. with a virus was significantly enhanced (*P* < 0.01) over that of uninoculated, RPMI-inoculated, or footpad-inoculated controls.

receptors for these lymphokines may inhibit the responses of the cells to them.

Because virus-induced peritoneal exudates and IFN- β (which is a constituent of these exudates) attracted LAK cells, we questioned whether LAK cells would selectively migrate into virus-infected tissue in vivo. To test this, we established infections in the peritoneal cavities of mice by i.p. inoculation with MCMV, MHV, LCMV, or VV. After 4 days of culture, LAK cells (3×10^6 /ml) were radiolabeled by addition of 1 μ Ci of 125 I-labeled iododeoxyuridine (Dupont, NEN Bioproducts, Boston, Mass.) for 24 h. At designated days post virus infection, 10^7 radiolabeled LAK cells were injected into the mice intravenously via the retro-orbital sinus. NK cells accumulate in the peritoneal cavities of mice infected with viruses i.p. but not by other routes (10). We therefore determined whether radiolabeled LAK cells would preferentially accumulate in the peritoneal cavities of virus-infected but not uninfected mice. At different times after LAK cell inoculation, the mice were sacrificed and exsanguinated. Peritoneal exudate cells were isolated by lavage of the peritoneal cavities with 4 ml of RPMI medium, and the cells were pelleted, centrifuge washed, pelleted again, and counted for radioactivity. Time course studies of 1, 2, 6, and 24 h indicated peak LAK cell accumulation in the peritoneal cavities at 2 to 6 h postinoculation of LAK cells. Significant increases in cell-associated counts were seen in the peritoneal cavities of virus-infected mice compared with uninocu-

lated or medium-inoculated controls. Figure 1A shows that a significant increase in LAK cell accumulation occurred as early as 1 day post MCMV infection ($P < 0.01$). Enhanced accumulation of LAK cells was shown for each virus tested, including MCMV, LCMV, MHV, and VV (Fig. 1B and C) ($P < 0.01$). A 3- to 8-fold increase in counts was usually seen in peritoneal exudate cells isolated from virus-infected mice, and the average increase in nine experiments with the various viruses at 3 days postinfection was 3.7-fold. This increase in cell-associated counts in the peritoneal cavities cannot be explained by host cells, such as macrophages, taking up radiolabel from degraded LAK cells, because ^{125}I is not reutilized in vivo after cell death; some of it concentrates in the thyroid, but the rest is rapidly secreted from the body (13). Inoculation of virus i.p. was important to stimulate LAK cell migration into the peritoneal cavity, since relatively few LAK cells migrated to the peritoneal cavities of mice inoculated i.p. with medium (Fig. 1B) or in the footpad with virus (Fig. 1C). We therefore interpret these experiments to mean that cells in LAK cultures will migrate into sites of virus infection after therapeutic administration.

These results show that cells in LAK cell cultures respond chemotactically to purified IFN- β and to virus-induced peritoneal exudates in vitro and preferentially migrate into virus-infected peritoneal cavities in vivo. In this respect, LAK cells resemble other types of activated lymphocytes which respond to similar chemotactic factors (11) and migrate into sites of inflammation (1, 8). These results, along with those of studies that demonstrated a marked prophylactic value of LAK cells delivered at the site of a virus inoculum (3), suggest that LAK cells should be examined for antiviral therapy, because an intravenous inoculum may provide an effective delivery system.

This work was supported by Public Health Service grants CA 34461 and AI 17672 and postdoctoral fellowship AI 307272 (to R.N.) from the National Institutes of Health.

We thank Carey O'Donnell for technical assistance and Mary Beth Aiello for preparation of the manuscript.

LITERATURE CITED

- Asherson, G. L., G. G. Allwood, and B. Mayhew. 1973. Contact sensitivity in the mouse. XI. Movement of T blasts in the draining lymph nodes to sites of inflammation. *Immunology* 25:485-494.
- Bukowski, J. F., J. F. Warner, G. Dennert, and R. M. Welsh. 1985. Adoptive transfer studies demonstrating the antiviral effects of natural killer cells in vivo. *J. Exp. Med.* 161:40-52.
- Bukowski, J. F., H. Yang, and R. M. Welsh. 1988. The antiviral effect of lymphokine-activated killer cells: characterization of the effector cells mediating prophylaxis. *J. Virol.* 62:3642-3648.
- 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.* 155:1823-1841.
- Kalland, T., H. Belfrage, P. Bhiladvala, and G. Hedlund. 1987. Analysis of the murine lymphokine-activated killer (LAK) cell phenomenon: dissection of effectors and progenitors into NK- and T-like cells. *J. Immunol.* 138:3640-3645.
- Koo, G. C., and C. L. Manyak. 1986. Generation of cytotoxic cells from bone marrow by human recombinant IL-2. *J. Immunol.* 137:1751-1756.
- Lotzova, E., and R. B. Herberman. 1987. Reassessment of LAK phenomenon: a review. *Nat. Immun. Cell Growth Regul.* 6:109-115.
- Moore, A. R., and H. G. Hall. 1983. Nonspecific entry of thoracic duct immunoblasts into intradermal foci of antigens. *Cell. Immunol.* 8:112-119.
- Mule, J. J., S. Shu, and S. A. Rosenberg. 1985. The anti-tumor efficacy of lymphokine-activated killer cells and recombinant interleukin-2 in vivo. *J. Immunol.* 135:646-652.
- Natuk, R. J., and R. M. Welsh. 1987. Accumulation and chemotaxis of large granular lymphocytes at sites of virus replication. *J. Immunol.* 138:877-883.
- Natuk, R. J., and R. M. Welsh. 1987. Chemotactic effect of human recombinant interleukin-2 on mouse activated large granular lymphocytes. *J. Immunol.* 139:2737-2743.
- Phillips, J. H., and L. L. Lanier. 1986. Dissection of the lymphokine-activated killer phenomenon. Relative contribution of peripheral blood natural killer cells and T lymphocytes to cytotoxicity. *J. Exp. Med.* 164:814-825.
- Riccardi, C., A. Santoni, T. Barlozzari, P. Puccetti, and R. B. Herberman. 1980. In vivo natural reactivity of mice against tumor cells. *Int. J. Cancer.* 25:475-486.
- Rosenberg, S. A., E. A. Grimm, M. McGrogan, M. Doyle, E. Kawasaki, K. Koths, and D. F. Mark. 1984. Biological activity of recombinant interleukin-2 produced in *Escherichia coli*. *Science* 223:1412-1415.
- Rosenberg, S. A., M. T. Lotze, L. M. Mude, S. Leitman, A. E. Chang, S. E. Ettinghausen, Y. L. Matry, J. M. Skibber, E. Shiloni, J. J. Vetto, C. A. Seipp, C. Simpson, and C. M. Reichert. 1985. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N. Engl. J. Med.* 313:1485-1492.
- Salup, R. R., B. J. Mathieson, and R. H. Wiltout. 1987. Precursor phenotype of lymphokine-activated killer cells in the mouse. *J. Immunol.* 138:3635-3639.
- Wang, A., S. D. Lu, and D. Mark. 1984. Site-specific mutagenesis of the human interleukin-2 gene: structure-function analysis of the cysteine residues. *Science* 224:1431-1433.
- Welsh, R. M. Natural killer cells and interferon. *Crit. Rev. Immunol.* 5:55-93.
- Yang, J. C., J. J. Mule, and S. A. Rosenberg. 1986. Murine lymphokine-activated killer (LAK) cells. Phenotypic characterization of the precursor and effector cells. *J. Immunol.* 137:715-722.