Mechanism of Cultured Endothelial Injury Induced by Lymphokine-activated Killer Cells

Dusan Kotasek, Gregory M. Vercellotti, Augusto C. Ochoa, Fritz H. Bach, James G. White, and Harry S. Jacob

Department of Medicine, Division of Hematology [D. K., G. M. V., H. S. J.], Immunobiology Research Center [A. C. O., F. H. B.], and Department of Laboratory Medicine and Pathology [J. G. W.], University of Minnesota, Minneapolis, Minnesota 55455

ABSTRACT

A new form of therapy of experimental tumors, utilizing lymphokine-activated killer (LAK) cells and high doses of interleukin 2, has recently been applied in the treatment of human malignancies. Several side effects suggestive of a diffuse vascular injury of unknown etiology, have prevented a more widespread application of this form of therapy. We have investigated the etiology of this clinical capillary leak syndrome, using an in vitro model of endothelial injury. LAK cells, but not interleukin 2 itself, are cytotoxic to cultured human endothelial cells, and this cytotoxicity is time and dose dependent. This human endothelial cell cytotoxicity can be inhibited by depletion of extracellular Ca2+, inhibition of the effector cell microtubular system, and inhibitors of serine proteases, but is not inhibited in the presence of toxic oxygen radical scavengers. LAK cell-mediated endothelial cytotoxicity is far more potent than that exhibited by maximally stimulated polymorphonuclear leukocytes. LAK cell-mediated injury of human endothelium may possibly be responsible for the capillary leak syndrome observed in patients treated with high doses of interleukin 2 and LAK cells.

INTRODUCTION

Peripheral blood human lymphocytes, if cultured for several hours in medium containing the lymphokine IL-2 become cytolytic for a wide spectrum of target cells. Such transformed lymphocytes, referred to as LAK cells (1), can lyse diverse cultured cell lines. Of potential therapeutic importance, LAK cells also lyse an array of fresh tumor cells in short-term chromium release assays (2–5), while generally not damaging normal, noncultured cells (6). Encouraged by this dichotomy, several trials—first in experimental animals and more recently in humans—have been undertaken to adaptively transfer in vitro-generated, autologous LAK cells back into subjects with malignant tumors as an alternative approach to use of specific viro-generated, autologous LAK cells back into subjects with malignant tumors as an alternative approach to use of specifically immune cells (7–9). This therapeutic approach is usually combined with the administration of high doses of IL-2 to the tumor-bearing individual—a maneuver thought essential for efficacy.

Recent clinical trials in which repeated infusions of LAK cells and IL-2 were administered, although modestly promising, have produced troublesome side effects (10–12), the most serious being an apparent CLS characterized by rapid weight gain, anasarca, pulmonary edema, hypoalbuminemia, and multiple organ dysfunction (10–12). The cause of the syndrome, which can require intensive-care-unit treatment, is enigmatic, although patients tend to improve rapidly after cessation of IL-2 infusion. In a murine model the syndrome is greatly attenuated if animals are pretreated with cyclophosphamide, steroids, or whole-body irradiation and is absent in nude (athymic) mice, suggesting that activated lymphocytes, themselves, are critically involved in its etiology (9, 13).

Our laboratory has had long-standing interest in mechanisms of endothelial cell injury in capillary leak disorders, such as the adult respiratory distress syndrome. Although our previous attention has centered on granulocytes as effector cells of vascular damage, in the present studies we have used our previously described (14) in vitro model of human endothelial monolayer injury to investigate whether LAK cells can provoke endothelial damage. Our findings indicate that LAK cells are indeed highly damaging to human endothelial monolayers and are far more toxic to endothelial cells than maximally stimulated granulocytes. This LAK cell-mediated cytotoxicity does not require the presence of IL-2, but directly reflects the number of added LAK cells and seems to require the presence of a LAK cell serine protease.

MATERIALS AND METHODS

Preparation of Endothelial Cells. HEC, obtained from umbilical cords by our modification (14) of the method of Jaffe et al. (15), were grown in 48-well 1-cm2 tissue culture plates (Costar, Cambridge, MA). Primary confluent monolayers, confirmed to be HEC by immunofluorescent staining with rabbit anti-Factor VIII antibody (Behring Diagnostics, La Jolla, CA), were used in cytotoxicity assays (see below). The average number of HEC per 1-cm2 well was approximately 100,000 (±10%, SE)/well in all experiments.

Generation of LAK Cells. After obtaining informed consent, heparinized blood was drawn from normal volunteers and from patients with solid tumor malignancies (renal carcinoma, colon carcinoma, and melanoma) who were undergoing LAK therapy at the University of Minnesota Clinical Research Center. PBMC were separated using Ficol-Hypaque density gradients (Histopaque 1077; Sigma, St. Louis, MO), and LAK cells were prepared according to previously described methods (3). In some experiments, PBMC were cultured for up to 7 days in culture medium devoid of IL-2.

Assays for LAK Activity and HEC Cytotoxicity. A 4-h 51Cr release assay was used for the assay of LAK activity as described previously (3). Target cells were the NK-sensitive cell line, K562, and the NK-insensitive cell lines, HL-60 and Daudi.

To determine endothelial cytotoxicity, we used confluent 4- to 6-day-old HEC growing in 1-cm2 culture wells as previously described (14). Briefly, following washing with assay medium, cells were labeled for 3 h (at 37°C) with 3 μCi/well of Na251CrO4 and washed, and effector cells (LAK cells or PBMC cultured in the absence of IL-2) were added to various E:T ratios; in some experiments effector cells were suspended in assay medium containing 1000 units/ml of IL-2. The culture plate was centrifuged (100 × g, 3 min) and incubated for various periods up to 18 h at 37°C, following which supernatants from centrifuged wells were removed and counted. The specific 51Cr release was calculated as described previously (14). In some experiments, confluent bovine aortic endothelial cells, passaged at least 20 times, were used as targets instead of HEC. Cytotoxicity was confirmed by light and electron microscopy as well as by trypan blue exclusion assays.

Several potential inhibitory substances of LAK cytotoxicity were...
added to LAK cells in the HEC cytotoxicity assays, including: colchicine (Eli Lilly, Indianapolis, IN); cytochalasin B (Aldrich, Milwaukee, WI); and superoxide dismutase and catalase (Sigma, St. Louis, MO). To investigate the role in cytotoxicity of LAK cell serine proteases, \(5 \times 10^6\) pelleted LAK cells were incubated for 30 min with the serine protease inhibitors PMSF (Sigma) or 3,4-DIC (Calbiochem, La Jolla, CA). Following the incubation, cells were washed 2 times, resuspended in fresh assay medium, and added to target cells. To determine calcium involvement in cytotoxicity, we first detached \(^{51}\text{Cr}\)-labeled monolayer HECs with 5 mM EDTA; these were washed, counted, and then added to U-bottomed microtiter wells together with LAK cells suspended in either \(\text{Ca}^{2+}\)-containing HBSS or in a \(\text{Ca}^{2+}\)-free medium (HBSS with 3.0 mM ethyleneglycol-bis(\(\beta\)-aminoethylether)-\(N,N,N',N'\)-tetraacetic acid added). Following brief centrifugation to promote target:effector cell contact, cytotoxicity was assayed as described above. No significant loss of LAK cell viability was detected with any of the perturbants. All experiments were performed in triplicate, and the results are expressed as mean ± SE of at least six separate experiments.

Serine Esterase Assay. Serine esterase activity of LAK cells after various durations of culture was assayed by the method of Pasternak and Eisen (16), using BLT as the chromophore.

Microscopic Studies. Culture wells containing endothelium with or without LAK cells were prepared for electron microscopic studies by fixing for 60 min in 3% glutaraldehyde prepared in White's saline (pH 7.3) according to previously published methods (17). Fixation was continued for 90 min in 1% OsO4 containing 1% potassium ferrocyanide. After exposure to the second fixative, samples were dehydrated in a graded series of alcohol and then flat embedded in Epon 812. Fragments of the culture well with its coating of Epon were broken off and cross-sectioned with an ultramicrotome. Contrast of ultrathin sections was enhanced with uranyl acetate and lead citrate.

Statistical Analysis. Experimental results were analyzed with the Student t test using the StatWorks computer software program.

RESULTS

Characteristics of LAK Cell-mediated Cytotoxicity. If not exposed to IL-2, PBMC do not become cytotoxic toward cultured HEC. That is, PBMC cultured for as long as 7 days in medium devoid of IL-2 and then added at E:T ratios of 5:1 cause no lysis of cultured HEC in 18-h assays (Fig. 1A); even if added at 40:1 E:T ratios, no PBMC-mediated injury was discernible (data not shown). In striking contrast these same cells, cultured for as little as 24 h in the same medium—but with recombinant IL-2 added (1000 units/ml)—provoked marked HEC cytotoxicity. Optimal monolayer disruption (approximately 30% cell lysis) occurs with IL-2-exposed cells cultured for 5 or more days (Fig. 1A). These LAK cells were also shown in parallel studies (not shown) to develop cytolytic potential against several cultured tumor cell lines (HL-60, K562, and Daudi) with a similar time course. Significant cytotoxicity to HEC remained perceptible if LAK cells were cultured with IL-2 for up to 32 days, and LAK cells generated from cancer patients were equally cytotoxic toward HEC as those derived from normal donors. Interestingly, human LAK cells were also cytotoxic to xenogenic endothelial cells; bovine endothelial cell monolayers underwent 61.1 ± 30.5% lysis at a 5:1 E:T ratio.

LAK cell cytolysis of HEC is both dose and time dependent. Significant toxicity occurs with E:T ratios as low as 0.5:1 and increases progressively with increasing LAK cell concentrations (Fig. 1B). At E:T ratios greater than 50:1, a plateau effect was noted with little additional cytotoxicity (approximately 75%) observable at ratios between 50:1 and 100:1 (data not shown). Using an intermediate E:T ratio of 5:1 we could detect HEC lysis after as little as 4 h of LAK cell exposure which reached 8.3 ± 0.1% at 10 h and 17.8 ± 2.9% by 18 h—our traditional assay interval.

Radiolabel release in these studies reflects mainly HEC lysis; that is, 90.2 ± 1.2% of supernatant radioactivity was soluble and not pelletable. Cytolysis was validated and followed microscopically. As seen in Fig. 2B, LAK cell-mediated damage to the endothelium is obvious, characterized by disruption and destruction of the endothelial monolayer compared to the intact endothelium shown in Fig. 2A. By electron microscopy (Fig. 2D), LAK cells cause HEC plasma membrane blebbing and cytoplasmic organelle disruption which is in sharp contrast to the intact appearance of control HEC monolayers (Fig. 2C).

In patients, side effects including CLS have previously been thought directly related to the concentrations of IL-2 infused; this has led to the suggestion that IL-2, itself, might be a direct cytotoxin. However, we could detect no HEC cytotoxicity in monolayers incubated with very high concentrations of IL-2—up to 7500 units/ml, a dose several times that extant in patients. Furthermore, preincubation of HEC with IL-2 (1000 to 6000 units/ml) for 24 h prior to the addition of LAK cells does not result in increased LAK cell cytotoxicity, nor is LAK cell-induced cytotoxicity rendered more efficient if IL-2 (100 units/
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cytokines and monocytes damage endothelium. A prominent role for toxic O$_2$ species, such as superoxide, hydrogen peroxide, and hydroxyl radical, has been implicated in the damage since it can be largely inhibited by addition of superoxide dismutase and catalase. A similar mechanism does not seem germane to LAK cell-mediated endothelial cytotoxicity, since neither superoxide dismutase (10 µg/ml) nor catalase (40 µg/ml), nor their combination inhibits cytotoxicity (107.3 ± 2.3% of control values). In contrast, prior investigations of others have demonstrated a different mechanism by which NK cells and cytotoxic T-cells (CTL) damage target tissues; that is, these cells secrete by exocytosis cytolytic substances stored in their prominent cytoplasmic granules (22–25). Since LAK cells share many characteristics with NK and CTL cells, we hypothesized they might utilize similar cytolytic mechanisms. In partial validation we have used several perturbants known to interfere with NK- and CTL-mediated killing and found identical interference in our LAK cell cytotoxicity assay system. Thus, divalent calcium is essential for both NK- and CTL-mediated cytolysis (26, 27), and we now report, for LAK cell aggression as well; LAK-mediated killing of HEC is largely prevented in Ca$^{2+}$-deficient medium (25.3 ± 5.1% in calcium-containing versus 3.8 ± 1.2% in calcium-free medium; P < 0.001). In addition, an intact microtubule system—required for granule exocytosis and previously demonstrated to be essential for the cytolytic activity of CTL (26, 27)—is also critical to LAK cell-mediated HEC cytotoxicity; that is, HEC damage is reduced by 36 ± 2 and 56 ± 4% with addition of the microtubule inhibitor, colchicine, at concentrations of 1 and 2 µM, respectively. Addition of the microfilament inhibitor, cytochalasin B (50 µg/ml), on the other hand did not affect LAK cell-mediated HEC cytotoxicity. Finally, others have shown that one or more serine proteases may be critical cytolytic constituents of cytoplasmic granules in NK and CTL cells (16, 28–30). To document an analogous involvement in LAK cell aggression we used two different serine protease inhibitors, PMSF or 3,4-DIC, in our HEC cytotoxicity assays. PMSF (1 mM or 5 mM) incubated with LAK cells prior to addition to HEC monolayers significantly reduces cytotoxicity (16% and 37%, respectively; P < 0.01). Even more potent is 3,4-DIC which, when added to LAK cells at concentrations as low as 60 µM for 30 min, completely inhibits LAK cell-mediated killing of HEC.

That granule serine protease(s) is indeed involved in LAK cell-mediated endothelial damage is also suggested by results shown in Fig. 3. Fresh PBMC which manifest minimal cytotoxicity toward HEC contain virtually no assayable serine protease activity as measured with the highly specific thiobenzyl

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**Fig. 2.** Morphology of LAK cell-HEC interaction. A, light microscopy (x 400) of a normal HEC monolayer stained with Wright’s stain. B, LAK cells added at a 5:1 E:T ratio to HEC for 18 h. The LAK cell nuclei appear dark and closely apposed to the larger HEC. The light microscopic studies were performed on the same day using the same primary culture of HEC monolayers. C, electron micrograph (x 10,000) of normal HEC monolayers. D, electron micrograph (x 10,000) of LAK cells (L) added to endothelial cells (E) for 18 h. The endothelial cell membrane has been breached (arrow), and the intracellular contents were disrupted.}

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**Fig. 3.** LAK cell BLT-reactive serine esterase activity and HEC cytotoxicity. LAK cells in culture for varying time periods display BLT-reactive serine esterase content (A) measured by $\Delta A_{414}$/10$^6$ LAK cells as described in "Materials and Methods." LAK cells (5:1 E:T ratio) were added to HEC, and specific $^{51}$Cr release was measured (C) as described in "Materials and Methods." Results are expressed as the mean of 6 experiments performed in triplicate.
earlier than peak serine protease activity. BLT-reactive serine ester substrate, BLT (A). However, serine protease content of analogous to that used by CTL and NK cells, and seems not been encountered in many clinical trials to date. From our cautious optimism attending this approach has been tempered exciting approach to the treatment of human neoplasia. The they remain 2 to 3 times less toxic to HEC than LAK cells (20.9 ± 1.8% and 56.2 ± 4.5%, respectively).

DISCUSSION

Adoptive immunotherapy that uses LAK cells and IL-2 administered to patients with malignancies represents a new and exciting approach to the treatment of human neoplasia. The cautious optimism attending this approach has been tempered by severe, dose-limiting toxicity, particularly CLS, which has been encountered in many clinical trials to date. From our studies we suggest that IL-2 may promote vascular damage by expanding and stimulating endogenous and adoptively transferred immune cells; these, in turn, injure the endothelium.

Damle et al. (31) and Miltenburg et al. (32) have published studies that also examined the cytotoxicity of LAK cells against normal human endothelial cells with similar results to ours. In the present studies we amplify these results in several ways: (a) we provide time courses of LAK cell development and note that the capability of LAK cells to damage endothelium develops extremely rapidly after exposure of lymphocytes to IL-2 (Fig. 1A); (b) we demonstrate that IL-2 is not itself cytotoxic for HEC even when added at very high concentrations; and (c) we show that the mechanism of LAK cell toxicity for HEC is different from that used by stimulated granulocytes and is far more potent.

In the latter regard, our laboratory has intensely investigated the mechanism by which granulocytes damage vascular endothelium. Unlike the case of neutrophil-mediated endothelial injury, where toxic oxygen radicals play a major role, LAK cell cytotoxicity is shown to involve a distinct killing mechanism, analogous to that used by CTL and NK cells, and seems not importantly to involve the generation of toxic oxygen species. Experimentally, four distinct steps have been identified in CTL and NK cell killing (26, 29): (a) receptor-mediated binding to targets; (b) polarization of Golgi apparatus, granules, and other cytoskeletal elements toward the target cell; (c) secretion/granule exocytosis; and (d) Ca²⁺-dependent assembly of transmembrane pores. Several of these steps are known to be dependent on an intact cytoplasmic microtubule system, temperature, and the presence of divalent cations, in particular Ca²⁺. We have shown that LAK cell cytotoxicity is very similar to that wrought by CTL and NK cells and is inhibited by agents which also inhibit CTL and NK cell killing: to wit, cytoplasmic microtubules appear to be critical since colchicine inhibits cytotoxicity of LAK cells as it does with CTL and NK cells (26, 27); extracellular Ca²⁺ is crucial to NK and CTL cytotoxicity and, we find, that in its absence LAK cytotoxicity is reduced by 85%; perhaps most importantly, serine proteases are found in the dense cytoplasmic granules of CTL, NK, and LAK cells, and one in particular, serine esterase I, has been shown to have significant proteolytic and cytolytic activity (16, 29, 30). A role for serine proteases in LAK cell-mediated HEC cytotoxicity seems likely in that PMSF and 3,4-DIC both significantly inhibit LAK-mediated cytotoxicity and serine esterase I, which reacts with the thiobenzyl ester substrate, BLT, and is released into the supernatant during the LAK killing process; moreover, normal uncultured peripheral blood lymphocytes contain minimal amounts of BLT-reactive serine esterase, but during incubation with IL-2 this activity increases in rough parallel with increasing LAK cell cytotoxicity (Fig. 3).

Activated lymphocytes, including LAK cells, have been shown in several studies (33, 34) to avidly bind to endothelial cells as the first step in their normal emigration from the circulation into both lymphoid and nonlymphoid tissues. Amplification of this adhesion may accompany local release of cytokines such as interleukin 1, tumor necrosis factor β, and γ-interferon by diverse inflammatory cells, including LAK cells themselves; these cytokines can affect the expression of endothelial cell surface molecules, such as the Class II HLA antigens, and alter concomitantly the endothelial cytoskeleton and membrane “stickiness” (35–38). Particularly relevant to the present studies, Cotran et al. (39) have recently shown that i.v. administration of IL-2 “activates” endothelial cells as discerned by their expression of neoantigens; moreover their studies demonstrate that exogenous IL-2 works by provoking release of endogenous cytokines which, in turn, alter endothelium so that it attracts inflammatory cells, eventually in vascular leak. In sum, their studies suggest that both IL-2 plus inflammatory cells are required for vascular damage to occur.

In support, although CLS that is provoked in human and animals studies improves rapidly following the cessation of infused high doses of IL-2, an additional critical role of mononuclear cells in the syndrome’s etiology is evident. For instance, despite administration of very high doses of IL-2, nude mice fail to develop CLS, yet the syndrome is observed in these animals following immune reconstitution. Moreover, the severity of CLS is diminished if experimental animals are treated prior to administration of IL-2 with cyclophosphamide, total-body irradiation, or corticosteroids—maneuvers known to decrease lymphocyte number (13). These observations plus the present data prompt the speculation that CLS observed in patients treated with IL-2 with or without LAK cells is caused by endogenously generated LAK cells as well as by those infused in vitro. We emphasize, however, that extrapolation of our in

![Graph](https://example.com/graph.png)

Fig. 4. HEC cytotoxicity mediated by PMN or LAK cells. Polymorphonuclear leucocytes (PMN) (40:1 E:T ratio) with or without phorbol myristate acetate (PMA, 100 ng/ml) or LAK cells (40:1 E:T ratio) were added to HEC, and specific ⁵¹Cr release was measured as described in “Materials and Methods.” LAK cells are significantly more toxic than maximally stimulated PMNs (P < 0.001). Columns, mean of 6 experiments performed in triplicate; bars, SE.
In vitro data to the CLS seen in patients should be cautiously, and we particularly acknowledge that previous studies by others have shown diverse cell lines are often insensitive to LAK cell injury unless cultured for finite periods. Thus the damage to endothelial cells noted in the present studies may to some unknown extent reflect culture-induced perturbations and be irrelevant to events involving the normal vasculature.

In any case, our studies demonstrate that LAK cells should be added to the list of inflammatory cells that can cause vascular endothelial injury. In fact, LAK cells appear to be far more potent than neutrophils in vitro, and we speculate that activated lymphocytes may effect endothelial damage in disease states such as vasculitis, organ rejection, and graft-versus-host disease, as well as in the more narrow arena of LAK cell immunotherapy considered herein.

REFERENCES


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