Susceptibility to Lysis of Pulmonary Alveolar Macrophages by Human Lymphokine-activated Killer Cells

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ABSTRACT

In this study we addressed the question of whether lymphokine-activated killer (LAK) cells, besides killing neoplastic cells, may exert a certain degree of lysis on the normal counterpart; in particular we took into consideration the toxicity against pulmonary alveolar macrophages (PAM). We demonstrated that human LAK cells generated in vitro following incubation of peripheral blood mononuclear cells with recombinant interleukin 2 for 4 days were able to lyse normal PAM in a 4-h aCr release assay. Similarly, PAM recovered from patients suffering from nonneoplastic interstitial lung disorders, i.e., sarcoidosis and hypersensitivity pneumonitis, were shown to be susceptible to the cytotoxic function provided by LAK cells. Both autologous and allogeneic PAM were lysed by LAK cells, thus suggesting that the phenomenon we observed does not require a major histocompatibility complex restriction. Preincubation of PAM under study with γ-interferon did not affect their susceptibility to the lysis mediated by LAK cells. Furthermore, cold target inhibition assay demonstrated that normal PAM could efficiently compete with both NK-sensitive and NK-resistant target lines for the binding sites on LAK cells, thus indicating that the putative receptor(s), or at least the mechanism of target recognition, is shared by PAM and these different target cell lines. The evidence herein provided that LAK cells are cytotoxic to normal, nontransformed PAM points out that the pathogenetic mechanisms involving this self-addressed lytic activity could account for some adverse reactions related to LAK/interleukin 2 immunotherapy.

INTRODUCTION

In vitro activation of normal PBMC with IL-2 generates non-MHC-restricted, highly cytotoxic lymphocytes which are referred to as LAK cells (1–4). These cells, without a previous sensitization, show a wide spectrum of cytotoxicity which encompasses NK-sensitive target cells (e.g., K562 cell line), NK-resistant targets (e.g., Daudi or Raji), and cell lines obtained from fresh or cryopreserved autologous and/or allogeneic tumors (2). By contrast, LAK cells have been supposed to spare the normal counterpart (1–3). LAK cells have recently triggered great interest in immunologists and oncohematologists because of their therapeutic possibilities in the treatment of cancer. In fact, the availability of IL-2 produced through the recombinant DNA technology, and then the facilities to generate large amounts of LAK cells in vitro, has enabled the exploration of potential benefits of this therapeutic approach in humans (5–7). Although promising responses have been observed by differ-
medium supplemented with 2 mmol glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (Flow Laboratories, Rockville, MD). Alveolar macrophages were obtained from the entire mononuclear cell suspension by two sequential adherence procedures at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 60 min in plastic Petri dishes and then removed using a disposable cell scraper as already described (21). The resulting cell populations were more than 95% pure on the basis of CD14 (LeuM3) antigen expression on fluorescence-activated cell sorter analysis. Aliquots of PAM were also stored in liquid nitrogen resuspended in 85% FCS plus 7.5% dimethyl sulfoxide. Nonadherent cells were represented by more than 95% of stored in liquid nitrogen resuspended in 85% FCS plus 7.5% dimethyl sulfoxide. Nonadherent cells were represented by more than 95% of lymphocytes, as determined by nonspecific esterase staining (less than 5% of cells were esterase positive). More than 95% of cells were viable, as judged by the trypan blue exclusion test. When cryopreserved cells were used, more than 90% of cells were viable after thawing, as evaluated by trypan blue exclusion test.

Preparation of Effector Cells. PBMC were obtained by centrifuging heparinized blood from normal donors over Ficoll-Hypaque gradient. The cells were washed three times in phosphate buffered-saline and were resuspended in RPMI 1640 (GIBCO, Paisley, Scotland) containing 10% FCS. Pulmonary lymphocytes were recovered from sarcoid and HP patients using the BAL technique, as described above. The average numbers of lymphocytes obtained from patients with sarcoidosis and HP were 133 ± 17 (SD) x 10⁶/ml and 199 ± 35 x 10⁶/ml, respectively. Unfortunately, the number of lymphocytes recovered from normal donors was not high enough to perform different cytotoxic tests in vitro (5.5 ± 1.1 x 10⁶/ml).

LAK cells were generated by culturing PBMC or BAL lymphocytes (10⁶/ml) with 100 units/ml of recombinant IL-2 (kindly provided by Glaxo Institute for Molecular Biology, Geneva, Switzerland) in RPMI 1640 supplemented with 10% FCS. Cells were optimally activated for 4 days in 25-cm² tissue culture flasks and were then washed twice in medium and readjusted to the original concentration. In some cases, BAL lymphocytes from sarcoid patients were cultured in IL-2 for up to 21 days, before LAK function testing. In these cases, cultures were expanded weekly by adjusting the cell concentration at 10⁴ cells/ml with fresh medium supplemented with 10% FCS and recombinant IL-2 (100 units/ml). At the end of the culture, LAK effectors were harvested and stored as reported above.

Cell Lines. The following tumor cell lines were used as targets in the cytotoxic assays: K562 (NK-sensitive) cells, Daudi and Raji (NK-resistant) cells, and Jok1 (NK-resistant) cells which were derived from a patient with hairy cell leukemia. Target cells were maintained in suspension culture in RPMI 1640 (Grand Island Biological Co., Grand Island, NY), supplemented with 4 mM glutamine, 1% penicillin-streptomycin, and 10% FCS. All cell lines were demonstrated to be free of Mycoplasma infection.

Cytotoxic Assay. A ⁵¹Cr release assay was used to measure the cytotoxicity of LAK effectors against autologous and allogeneic PAM, the NK-sensitive K562 and the NK-resistant Daudi, Raji, and Jok1 target cells (24). PAM were labeled with 400 μCi of sodium ⁵¹Cr-chromate (CEA IRE Sorin; Biomedica, Salugia, Italy) for 4 h at 37°C, extensively washed three times before use, and then adjusted to a final concentration of 10⁴ cells/ml in RPMI 1640 supplemented with 10% FCS. Raji, Daudi, and Jok1 cells were labeled with 200 μCi of ⁵¹Cr x 10⁶ cells for 2 h before the assay. To perform the test, 100 μl of labeled target cells (10⁴) were added to the effector cells, resulting in an effector to target ratio ranging from 40:1 to 5:1, in a final volume of 0.2 ml in each well. After a 4-h incubation at 37°C, the culture supernatants were harvested by collecting 100 μl of supernatant to be counted in a gamma counter. In some cases, effector and target mixtures were incubated for 18 h before evaluating ⁵¹Cr release. Spontaneous and maximum releases were measured by counting supernatants of targets incubated with medium alone and Nonidet P-40 detergent, respectively. Spontaneous release never exceeded 10% of the maximum value for K562, Raji, Daudi, and Jok1 cell lines, while spontaneous release for PAM was less than 20% in a 4-h assay and less than 35% in an 18-h assay. The mean value of triplicate assays was used to calculate the percentage of cytotoxicity according to the formula

\[
\% \text{ of cytotoxicity} = \frac{\text{cpm release in test} - \text{cpm spontaneous release}}{\text{cpm maximum release} - \text{cpm spontaneous release}} \times 100
\]

The standard error of the mean (SEM) of all assays was calculated and was less than 5%.

Pretreatment with IFN-γ. In some cases, normal PAM were incubated with recombinant IFN-γ (1000 units/ml; Boehringer Mannheim, Mannheim, Federal Republic of Germany) for 2 or 8 h before the test was accomplished. No differences were demonstrated for the spontaneous and maximum ¹⁵⁵Cr release of IFN-γ-treated and untreated PAM.

Cold Target Competition Assay. To verify whether the same determinants recognized by LAK cells were shared by normal PAM, K562, and Raji cell lines, a cold target inhibition assay was performed. A fixed number (10⁴) of ¹⁵⁵Cr labeled PAM (hot) was mixed with unlabeled (cold) PAM, K562, and Raji cells at the ratio of 10:1 or 1:1 cold to hot target. A similar experiment design was followed using the K562 or Raji cells as the hot target. All tests were performed at the constant ratio effectors to hot targets of 10:1. The results were expressed as percentage of inhibition, according to the formula

\[
\% \text{ of inhibition} = \left(1 - \frac{\% \text{ of release in the presence of inhibitor}}{\% \text{ of release without inhibitor}}\right) \times 100
\]

Phenotype of LAK Effector Cells. Phenotypic analysis of effector cells was performed using fluorescein- or PE-conjugated monoclonal antibodies and quantitated using a flow cytometer (Cytotfluorograph II; Ortho Diagnostic). Monoclonal antibodies used were CD3 (OKT3), CD4 (OKT4), CD8 (OKT8) (Ortho Pharmaceuticals); CD57 (anti-Leu7), CD56 (anti-Leu19), and HLA-DR (Becton-Dickinson). CD25 (anti-Tac) monoclonal antibody was kindly provided by Dr. T. Uchiyama, Kyoto, Japan. The CD nomenclature was reported according to the Fourth Standardization Meeting of Leucocyte Markers (25). Briefly, cells at a concentration of 10⁴/ml were incubated with the above quoted FITC- or PE-conjugated antibodies for 30 min in ice and then centrifuged twice in cold phosphate-buffered saline. For indirect fluorescence, a FITC-conjugated F(ab')² goat anti-mouse immunoglobulin (Techno Genetics, Turin, Italy) was added; cells were further incubated for 30 min at 4°C and washed twice. After these incubations, 10,000 gated cells were analyzed using the flow cytometer equipped with an argon laser operating at a wavelength of 488 nm to excite FITC and PE. Forward and 90-degree-angle light scatter were used to discriminate viable from dead cells. FITC and PE mouse immunoglobulins of the same isotype (IgG1, IgG2a+b) were used as negative controls.

Statistical Analysis. Data are expressed as mean ± SEM and comparisons between values made using the Cochran-Cox analysis. P < 0.05 was accepted as significant.

RESULTS

Generation of LAK Activity. Incubation of normal PBMC with IL-2 for 4 days induced the generation of high cytotoxic activity against the NK-sensitive K562 (mean ± SEM, 75 ± 6%) and the NK-resistant Daudi, Raji and Jok1 cell lines (86 ± 5%, 88 ± 4%, 34 ± 6%, respectively; results are referred to the 40:1 E:T ratio) (Fig. 1). PBMC obtained from patients with sarcoidosis and HP showed LAK activities consistent with those observed in normal controls (in both cases, the P values were not significantly different compared to controls).

BAL lymphocytes were shown to exhibit LAK function following IL-2 activation, even if at a lower level than PBMC values (at the 40:1 E:T ratio against Daudi targets: sarcoid lymphocytes, 42 ± 10%; HP lymphocytes, 52 ± 5%) (Fig. 2). As reported in Fig. 2, a low but detectable level of LAK activity against the Daudi targets was exhibited by HP lymphocytes at resting conditions, as reported previously (26). Sarcoïd BAL
lymphocytes at resting conditions were not cytotoxic against the target used.

**PAM Lysis by LAK Effectors.** Fig. 3 shows that LAK cells were able to lyse PAM recovered from normal individuals (Fig. 3A) and from sarcoid (Fig. 3B) and HP patients (Fig. 3C). As shown in Fig. 3A, normal PBMC at resting conditions did not show any detectable level of cytotoxicity against PAM, while both normal autologous and allogeneic PAM were lysed by LAK cells with some efficiency. PBMC-derived LAK cells from sarcoid \((n = 4)\) and HP \((n = 3)\) patients showed a similar pattern \((22 \pm 7\% \text{ and } 22 \pm 4\%, \text{ respectively, against autologous PAM};\) \(28 \pm 6\% \text{ and } 24 \pm 7\%, \text{ respectively, against allogeneic PAM};\) results are referred to the 40:1 E:T ratio).

LAK activity generated from BAL lymphocytes also demonstrated a significant level of cytotoxicity against PAM (Fig. 4). According to the results obtained using LAK cells generated from PBMC, the LAK cells generated from BAL lymphocytes did not show MHC restriction. At resting conditions, BAL cells did not exhibit detectable levels of cytotoxicity against PAM.

Due to the very low number of lymphocytes recovered from normal donors, it was not possible to test the cytotoxic potential of normal BAL lymphocytes.

In 4 sarcoid cases we were able to evaluate the cytotoxicity of LAK effectors generated from BAL lymphocytes versus PAM following a 21-day culture period in IL-2. A representative experiment is reported in Fig. 5. Following 21 days of incubation with IL-2, LAK cells lost their ability to lyse autologous PAM, while a well demonstrable level of cytotoxicity was still present against the K562 and Daudi targets.

**Effect of IFN-γ Treatment on the Susceptibility of PAM to LAK Lysis.** In the majority of cases tested, normal autologous PAM lysis by LAK effector did not seem to be influenced by preincubation with IFN-γ (Table 1). Similar data were obtained also from BAL generated by BAL lymphocytes (data not shown). These results were reproduced in many different experiments with the exception of 2 cases (one of them reported in Table 1), in which a protective effect of the lysis was demonstrated.

**Cold Target Competition Assay.** As reported in Table 2, nonradiolabeled (cold) normal autologous PAM inhibited the lysis of hot PAM, K562, or Raji cells in a dose-dependent manner. A similar trend was also demonstrated using the K562 or the Raji cells as cold target.

**Phenotype of Effector Cells.** Table 3 shows the cell surface markers on resting and IL-2-activated effector cells obtained from either PBMC of normal individuals or BAL lymphocytes of sarcoid patients. Regarding the expression of CD3, CD4, CD8, CD16, and CD56 antigens, no major differences were found in PBMC LAK effectors with respect to the baseline conditions while a significant increase in CD25 and HLA-DR-positive cells was demonstrated. Following IL-2 stimulation, the percentage of BAL cells expressing the CD3, CD4, CD57, HLA-DR, and CD25 antigens was markedly higher with respect to the value observed at resting conditions. Using the double staining technique, most CD3+ LAK cells from PB were not demonstrated to express the CD16 and CD56 antigens. LAK cells obtained from BAL lymphocytes of sarcoid patients did coexpress CD3, CD4, and CD57 antigens, while the CD56 marker was lacking in most CD3+ lymphocytes. In contrast to PB lymphocytes, CD16 antigen was present on the surface of BAL lymphocytes in a very low percentage, both at resting conditions and following IL-2 activation.

**DISCUSSION**

In this study we provide evidence that LAK cells generated in vitro are cytotoxic to normal, nontransformed PAM. The lytic activity of LAK cells was not restricted to the MHC, since both autologous and allogeneic PAM were efficiently lysed. In addition, in the majority of cases, the pretreatment of PAM with IFN-γ did not protect them from lysis by LAK cells. The cold target competition assay suggested the possibility that LAK cells recognize the same receptor(s) on PAM, K562, and Raji cells.

The possibility that normal cells could be efficiently lysed by LAK cells must be taken into account during immunotherapy with LAK/IL-2 and with IL-2 alone, which is supposed to generate LAK activity in vivo (6). In particular, normal PBMC, endothelial cells, monocytes, and keratinocytes have been demonstrated to be susceptible to lysis by LAK cells (12–15). Our results extend this observation to pulmonary macrophages and suggest that some adverse effects of LAK therapy could be related to the cytotoxic action of LAK cells exerted outside the
blood stream. In this regard, it is worth mentioning that Lotze et al. and Ettinghausen et al. demonstrated both in mouse and in human models that, during immunotherapy with IL-2 and LAK cells, infused lymphocytes were located and preferentially proliferated in the lungs (17, 18). Our results suggest the possibility that lysis of PAM might represent not only an in vitro speculative model but could actually occur in vivo. The issue is further intrigued by the evidence of an important relationship between human PAM and LAK cells (18, 26). In fact, normal PAM were demonstrated to down-regulate the induction of LAK activity from PBMC when added at the beginning of the culture, while peripheral blood monocytes increased the generation of LAK function (27).

When we evaluated the lytic potential of LAK cells obtained from BAL lymphocytes following a 21-day culture in IL-2, we observed that the lytic activity against both NK-sensitive and NK-resistant target cells was nearly unchanged, while LAK cells were no longer cytotoxic to PAM. These preliminary data support the concept that the self-addressed cytotoxicity against the autologous cells is not a definitive function of LAK effectors but rather represents a transient property, with the ability to kill neoplastic cell lines being unchanged. Our results might also suggest that different subsets of LAK effectors with a different spectrum of cytotoxicity may develop during the culture in the presence of IL-2. In this regard, Peace and Cheever (28) on the basis of the demonstration in mice that the selective depletion of the NK cell subset reduces the toxicity and increases the therapeutic efficiency of IL-2, recently suggested that different subsets of lymphocytes could selectively mediate...
potentially toxic and therapeutic efficacy during IL-2 immunotherapy. Interestingly enough, the phenotype of the LAK cells generated from BAL lymphocytes is consistent with a population devoid of strictly related NK markers (see below).

Our data demonstrate that PAM lysis is not a MHC-restricted phenomenon since the lytic activity provided by LAK cells does not discriminate between autologous and allogeneic targets. Although the mechanisms of target recognition and the putative antigen(s) recognized by LAK effectors have not been identified (29), this piece of information adds further support to the concept that the tissue damage of a nonspecific cytotoxic effectors. The demonstration we provided that a LAK activity anti-PAM can be generated from pulmonary lymphocytes suggests a possible role in vivo of autologous LAK cells. The evidence that, in patients with HP, a certain level of LAK activity was exhibited by freshly recovered BAL lymphocytes (Table 1), suggests that a similar mechanism is already working in the lung in some reactive conditions (26). The reason for the appearance of anti-PAM activity in LAK cells is not clear. One possible explanation could rest on the presence of a feedback mechanism which reduces the in situ proliferation by down-regulating the production of interleukin 1 by PAM, when an excess of IL-2 is present. Further studies are needed to test this hypothesis.

The protective effect of IFN-γ on the lysis of tumor and normal cells by NK and LAK cells have been demonstrated in different studies (15, 30-33). However, in the majority of our cases, both IFN-γ-treated and untreated PAM were equally susceptible to the in vitro lysis. A tentative interpretation of this finding could be related to the peculiar role of alveolar macrophages during IL-2 stimulation. Since cells lining the lower respiratory tract are in direct contact with the external environment, it is tempting to speculate that a low amount of IFN-γ could be physiologically produced to maintain a subliminal but discrete level of cell activation. In accordance with this interpretation is the evidence that most PAM express class II MHC antigens (34, 35). Since the IFN-γ-mediated protection lasts for some days in the absence of IFN-γ (20), in vitro-cultured PAM should be more susceptible to lysis by LAK cells than uncultured PAM. Preliminary studies indicate that lysis of PAM cultured for 5 days in medium alone was higher than that of fresh PAM (data not shown). Alternatively, it is possible that a longer period (more than 8 h) of IFN-γ incubation or higher concentrations are needed in order to achieve a detectable level of cellular protection.

Phenotypic analysis of LAK effectors obtained from PBMC shows a heterogeneous pattern and is consistent with data reported by other authors (2, 36). In fact, the majority of peripheral blood effector cells mediating LAK function have been demonstrated to express the CD16 and CD56 antigens, thus suggesting that the majority of LAK activity observed in BAL lymphocytes is mediated by CD3+ cells. Since most lymphocytes present in the normal lung express the CD3 and CD44 molecules, it could be hypothesized that anti-PAM cytotoxic activity might be generated from normal BAL T-lymphocytes. In view of the consideration discussed above on the discrete ability to mediate toxicity and efficacy by different LAK cell populations (28), studies are now in progress to determine the source of cytotoxicity, rather than reflecting competitive binding of receptors.

The demonstration we provided that a LAK activity anti-PAM can be generated from pulmonary lymphocytes suggests a possible role in vivo of autologous LAK cells. The evidence that, in patients with HP, a certain level of LAK activity was exhibited by freshly recovered BAL lymphocytes (Table 1), suggests that a similar mechanism is already working in the lung in some reactive conditions (26). The reason for the appearance of anti-PAM activity in LAK cells is not clear. One possible explanation could rest on the presence of a feedback mechanism which reduces the in situ proliferation by down-regulating the production of interleukin 1 by PAM, when an excess of IL-2 is present. Further studies are needed to test this hypothesis.

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substantiate this hypothesis and to characterize precursor cells responsible for LAK activity against PAM.

In conclusion, our data support the concept that a self-directed cytotoxicity against PAM is generated during lymphocyte activation with IL-2. Further studies are needed to verify whether this phenomenon plays an actual role in vivo and, if this is the case, to evaluate the biological consequences of PAM lysis and eventually the possibility to manipulate LAK cells in order to either block or reduce the consequent damage of normal pulmonary parenchyma.

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REFERENCES


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