Inhibition of Lymphokine-activated Killer Cell Function by Human Alveolar Macrophages

Michael D. Roth and Sidney H. Golub

Divisions of Pulmonary Medicine [M. D. R.] and Surgical Oncology [S. H. G.], UCLA School of Medicine, Los Angeles, California 90024

ABSTRACT

Tissue- and organ-specific factors may be important in the regulation of cytotoxic lymphocytes. We therefore examined the ability of human alveolar macrophages (AMs) to alter the tumoricidal function of lymphokine-activated killer cells (LAK cells). AMs, obtained by bronchoalveolar lavage from healthy volunteers, or peripheral blood monocytes, were added to a standard 4-h chromium release LAK assay at varying concentrations. AMs severely inhibited the killing of both NK-sensitive (K562) and NK-resistant (M14) tumor cells [42 ± 2.6% (SEM) inhibition of M14 killing at the 0.125:1 AM:LAK ratio and 83 ± 2.3% inhibition at the 1:1 ratio, n = 9]. Peripheral blood monocytes, in contrast, were only one-eighth as inhibitory as AMs. A positive smoking history was associated with a 3- to 7-fold increase in the number of AMs recovered by bronchoalveolar lavage but had no effect on the inhibition produced per AM cell. The mechanism of inhibition was investigated. Formalin fixation produced an 8-fold reduction in the inhibitory capacity of AMs, suggesting the need for active metabolism or an intact cell membrane. No soluble mediator could be detected with a two-chamber Transwell system, in 24-h AM culture supernatants, or following blocking experiments with indomethacin, catalase, or superoxide dismutase. Binding studies demonstrated selective binding between LAK cells and AMs, yet AMs were not susceptible to LAK-mediated lysis under the usual assay conditions. In summary, AMs are potent inhibitors of in vitro LAK function. Inhibition requires direct cell-cell contact and is independent of soluble reactive oxygen species, prostaglandins, or activation by tobacco smoking. Inhibition is not due to lysis of the AM as a competitive cold target. These results suggest that AMs may actively limit antitumor cytotoxic responses in the lung.

INTRODUCTION

LAK cells are potent cancer-killing agents produced when lymphocytes are incubated with the lymphokine IL-2 (1). Interest in LAK cells and other tumoricidal lymphocytes has centered around their role in cancer defense (2, 3) and their utility as a form of cancer immunotherapy (4). Rosenberg et al. (4), infused autologous LAK cells and IL-2 into 106 patients with end stage metastatic tumors and achieved a partial or complete remission in 22%. Despite these advances, little is known about the tissue interactions and local regulation of transfused LAK cells. The lung provides a model environment for study. Not only is the lung involved in more cancer deaths than any other site (5), but it also contains a unique cell, the pulmonary macrophage. This cell, in addition to its well established phagocytic and bacteriocidal functions (6), has been described to inhibit the activation and effector functions of some cytotoxic lymphocytes (7-11). Bordignon et al. (7) first demonstrated the unique capacity of AMs, as compared to other monocytes and macrophages, to inhibit tumor killing by NK cells. These results have been confirmed by several investigators, with both AMs and tissue extracted pulmonary macrophages, and subsequently correlated to a marked deficiency of NK cell function in the lung (9, 10). The extent to which LAK cells are inhibited by AMs, or the functional status of LAK cells in the lung environment, has not been previously studied. There are several differences between LAK cells and NK cells which warrant their independent study: (a) it is unclear if LAK cells represent an activated/differentiated form of NK cell or an independent line of cytotoxic lymphocyte (1, 12, 13); (b) LAK cells kill a much broader range of tumors, with a much higher cytotoxic activity (10- to 100-fold higher), than NK cells and may therefore be resistant to suppression (1); (c) LAK cells have the potential to lyse NK-resistant lung cancers and offer a promising form of immunotherapy if active in the lung environment (14). Whether pulmonary macrophages can inhibit LAK activity is of further relevance since 50 to 70% of adoptively transferred LAK cells temporarily aggregate in the lungs before reaching a tumor site (15). This investigation describes the capacity of human AMs, as obtained by BAL (16, 17), to inhibit the in vitro tumoricidal function of LAK cells. We also evaluate the mechanism of inhibition, including the role of soluble mediators, such as reactive oxygen species and prostaglandins, and the importance of direct cell-to-cell interactions. Finally, we compare the inhibitory capacity of AMs to that of PBMs, their putative blood precursor, and to AMs from tobacco smokers, a group which has both an increased cancer risk and in vivo AM activation (18, 19).

MATERIALS AND METHODS

General Experimental Design. LAK cells were generated by culturing nonadherent PBLs in medium containing IL-2 for 4 days as described. On day 4, LAK cells, either alone or following the addition of potential inhibitors, were assayed for their ability to lyse tumor targets in a 4-h chromium release assay. The potential inhibitors, AMs, PBMs, or PBMs, were harvested from either BAL fluid (AMs) or peripheral blood (PBMs). LAK cells were harvested on the morning of the chromium release assay. LAK cells and inhibitors were either autologous or allogeneic, inasmuch as the source of LAK cells did not affect the results (8).

Reagents. Complete medium designates RPMI 1640 with glutamine (Flow Laboratories, McLean, VA) which was supplemented with 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, antibiotic-antimycotic mixture (GIBCO, Grand Island, NY), and 10% heat-inactivated and filtered human AB serum (Irvine Scientific, Santa Ana, CA). Recombinant IL-2, an alanine-125 analogue, was kindly provided by Amgen, Inc. (Thousand Oaks, CA). Indomethacin (Sigma, St. Louis, MO) was dissolved in absolute ethanol and diluted in complete medium prior to use. The final ethanol concentration of 0.01% had no effect on LAK activity. Lyophilized bovine liver catalase (Behring Diagnostics, La Jolla, CA) and lyophilized bovine erythrocyte SOD (Boehringer Mannheim, West Germany) were reconstituted fresh before each use.

Bronchoscopy. Healthy subjects, ages 20 to 45 years, with no history of pulmonary disease or recent respiratory tract infection were recruited to undergo outpatient fiberoptic bronchoscopy and BAL as described.
The percentage of lysis at each E:T ratio was plotted by an exponential curve, as described by Pross et al. (20) to determine the LU capacity of the effectors. One lytic unit represents the number of effector cells required to lyse 30% of the targets. LU values are reported as the number of LUs per 10^6 lymphocytes.

**RESULTS**

Inhibition of LAK Cytotoxicity by AMs. AMs, when added directly into the 4-h LAK assay, dramatically inhibited tumor lysis in a dose-dependent manner at every effector:target ratio (Fig. 1). The amount of inhibition, in terms of percentage of lysis, varied from one E:T ratio to another. Conversion to lytic unit values (LU/10^6 effector cells) allowed for a more direct comparison of inhibition within and between assays (Fig. 1). AMs suppressed the killing of both NK-sensitive (K562) and NK-resistant (M14) targets, but the effect was always greater with the M14 tumor line (n = 9, P ≤ 0.01). AMs produced 41.7 ± 2.6% inhibition of M14 killing at the 0.125:1 AM:LAK ratio which increased to 83 ± 2.3% inhibition at the 1:1 AM:LAK ratio.
INHIBITION OF LAK BY ALVEOLAR MACROPHAGES

Fig. 1. Inhibition of LAK cytotoxicity by AMs. (Left) The cytotoxicity inhibition assay was performed at four E:T ratios with either medium alone (0:1 AM:LAK ratio) or with increasing ratios of AM:LAK (0.125:1, 0.25:1, 0.5:1, and 1:1). Results are presented in percentage of lysis (SEM ± 2.5%) for a single experiment. (Right) Conversion of the data to LU values at each AM:LAK ratio demonstrates that both M14 and K562 target killing were severely inhibited by the presence of AMs (n = 9). Inhibition was always greater with M14 (P < 0.01). LU control values were 104 ± 14 LU (M14) and 86 ± 13 LU (K562).

Fig. 2. Inhibitory capacity of AMs from smokers versus nonsmokers. There was no significant difference (P = 0.52) in the inhibitory capacity of AMs from 5 healthy smokers, as compared to 5 healthy nonsmokers, at any AM:LAK ratio. The addition of PBLs had a minimal effect on tumor killing (maximum inhibition, 17.2 ± 1.2%, n = 6), while the addition of PBMs did inhibit tumor killing (P ≤ 0.01, n = 6). PBMs, however, were not more potent than formalin-fixed AMs. The maximum inhibition produced by PBMs, 47.2 ± 5.2%, was equal to that produced by one-eighth as many fresh AMs (0.125:1 ratio).

Mechanisms of LAK Inhibition. Potential mediators of AMs, reactive oxygen species and prostaglandins, were manipulated in vitro to determine their role in AM-mediated inhibition (Fig. 4). Pharmacological blockade with 2 μg/ml indomethacin, 2500 units/ml catalase, or 1000 units/ml SOD had no effect on the capacity of AMs to suppress LAK cytotoxicity (P = 0.96, n = 3). The further possibility of a soluble inhibitor was evaluated with two different techniques. First, 24-h AM culture supernatants were tested for inhibitor activity (n = 3). Next, the presence of a labile or low concentration inhibitor was evaluated by using a porous membrane system (Transwell) to separate AMs from the effector and target cells during the 4-h assay (n = 3). In neither case was there any detectable attenuation of LAK activity (0.3 ± 5.4 and 0.0 ± 5.7% inhibition, respectively).

Binding Characteristics of LAK Cells, AMs, PBMs, and PBLs. Single cell binding studies were performed to measure the cell-to-cell interaction between LAK cells, AMs, PBMs, or...
INHIBITION OF LAK BY ALVEOLAR MACROPHAGES

Fig. 4. Effect of prostaglandin and reactive oxygen blockade on the inhibitory capacity of AMs. The LAK inhibition assay was performed in the presence of either complete medium, 2 μg/ml indomethacin, 2500 units/ml catalase, or 1000 units/ml SOD. These agents had no effect on baseline LAK activity (0:1 E ratio) or its inhibition by AMs (P = 0.96, n = 3). Control; (+) indo; (+) catalase; (+) SOD. Bars, SEM.

Table 1 Tumor binding capacity of LAK cells, AMs, PBMs, and PBLs

<table>
<thead>
<tr>
<th>Binding pair</th>
<th>% bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAK:M14</td>
<td>44.8 ± 2.2</td>
</tr>
<tr>
<td>LAK:K562</td>
<td>46.7 ± 1.3</td>
</tr>
<tr>
<td>AM: M14</td>
<td>35.3 ± 3.9</td>
</tr>
<tr>
<td>AM: K562</td>
<td>40.5 ± 3.2</td>
</tr>
<tr>
<td>PBM: M14</td>
<td>41.5 ± 6.3</td>
</tr>
<tr>
<td>PBM: K562</td>
<td>41.8 ± 4.4</td>
</tr>
<tr>
<td>PBL: M14</td>
<td>8.8 ± 0.9</td>
</tr>
<tr>
<td>PBL: K562</td>
<td>15.5 ± 1.3</td>
</tr>
</tbody>
</table>

PBLs and the two tumor targets (Table 1). As expected, LAK cells bound both tumor targets with a high frequency: 46.7 ± 1.3% bound K562 and 44.8 ± 2.2% bound M14 (n = 6). When AM or PBM binding was measured at 4°C there was little interaction with the tumor cells (data not shown). However, when measured at 37°C both AMs and PBMs bound tumor targets with a similarly high frequency: 40.5 ± 3.2% of AMs and 41.8 ± 4.4% of PBMs bound K562; 35.3 ± 3.9% of AMs and 41.5 ± 6.3% of PBMs bound M14 (n = 4). There were, therefore, no significant differences in the tumor-binding capacities of AMs and PBMs (P = 0.55). PBMs, by comparison, demonstrated only 15.5 ± 1.3% binding of K562 and 8.8 ± 0.9% binding of M14. These levels are consistent with binding by NK cells (n = 4) (23).

Single-cell binding studies were also performed to measure the interaction between LAK cells and either AMs, PBMs, or PBLs (Fig. 5). Interestingly, there were significant differences in the frequency of LAK cell binding to the different leukocyte populations. LAK cells (17.3 ± 3.6%) bound AMs, whereas only 7.8 ± 1.0% bound PBMs and only 3 ± 0.6% of LAK cells bound PBLs (P = 0.05, n = 4). The ability of these inhibitors to bind LAK cells was proportional to their ability to inhibit LAK function.

LAK Target Specificity Assay. We directly tested the sensitivity of chromium labeled AM, PBM, PBL, M14, and K562 targets to lysis by LAK cells (n = 3). Fresh PBL were able to lyse only K562 targets, which are NK-sensitive, despite E:T ratios up to 200:1 (data not shown). Following exposure of LAK cells to IL-2 we noted the progressive development of cytotoxicity against all of the targets with maximal activity on day 4 (Fig. 6). The lysis of AM, PBM, and PBL targets, while measurable, required E:T ratios 200 to 400 times greater than that required to lyse the M14 and K562 targets. At the E:T ratios utilized in the LAK inhibition assay, 1.25:1 up to 10:1, there was no measurable lytic activity against any of the fresh leukocyte targets.

DISCUSSION

Lung cancer is the number one cause of cancer deaths in the United States and its incidence will continue to increase over the next decade (5). Conventional surgery and chemotherapy have had little impact on these statistics. Immunotherapies with IL-2, LAK cells, and tumor-infiltrating lymphocytes (24) offer promising new approaches to treatment. Our understanding of those host factors which interact with and regulate cytotoxic cells will be important in improving the success of adoptive immunotherapy. In this report we demonstrated a profound inhibitory effect of AMs on the in vitro cytotoxic function of autologous and allogeneic LAK cells. The 10- to 100-fold higher cytotoxic activity of LAK cells, as compared to NK cells, did...
not allow them to escape the suppressive influence of AMs. In fact, the NK-resistant tumor target, a category into which most primary lung tumors fall, was significantly more sensitive to inhibition than the NK-sensitive one. The magnitude of AM-mediated inhibition is impressive, especially in light of the fact that AMs constitute 90 to 98% of the normal leukocyte population of the lung (17). The addition of only 12.5% AMs to the LAK assay inhibited tumor killing by 42% and an equal number of AMs (AM:LAK ratio, 1:1) inhibited tumor killing by 83%. The in vivo implications of these findings remain to be tested, but there is evidence to suggest that local inhibition of LAK activity occurs in the lung. Maghazach et al. (15) have demonstrated that 50 to 70% of adoptively transferred LAK cells localize in the lungs during the first 4 to 24 hours of treatment. Lung biopsy and BAL analysis in patients receiving IL-2 expanded tumor-infiltrating lymphocytes have also shown diffuse infiltration of the pulmonary interstitium and alveolar spaces with lymphocytes (24). Despite this deposition of cytotoxic effectors within the lung and their documented in vitro ability to lyse most fresh pulmonary tumors (14), primary lung cancer remains the least responsive tumor in adoptive immunotherapy trials (4, 24, 25).

Bordignon et al. (7) first observed that AMs were unique, as compared to other monocytes/macrophages, in their ability to inhibit NK cell function. Others have found that PBMs have similar, but much weaker, inhibitory properties (10). We tested the specificity of LAK suppression by comparing the inhibitory capacity of fresh AMs to that of several leukocyte populations: PBLs; formalin fixed AMs; or fresh PBMs. Nonadherent PBLs acted as a negative control, demonstrating the minimal effect produced by the addition of even large numbers of "nonspecific" functional cells to the LAK assay. Formalin fixation produced an 8-fold reduction in the inhibitory capacity of AMs, demonstrating that inhibition required either a metabolically active cell and/or an intact cell membrane. The residual activity seen with formalin fixation is interesting and may represent residual active membrane structures or steric hinderance. PBMs are putative precursors of the AM (26), and we were anxious to examine their effect on LAK cell function. PBMs did inhibit LAK function, but to a much smaller degree than that seen with AMs. The inhibition produced by a 1:1 ratio of PBM:LAK was only equivalent to that seen with one-eighth as many fresh AMs (0.125:1 ratio). These results suggest that mononuclear phagocytes have an innate inhibitory effect on the activity of LAK cells and that differentiation or environmental alteration of the macrophage occurs in the lung to enhance this inhibitory effect.

The mechanism(s) by which AMs inhibit cytotoxic cell function has remained elusive. The AM secretes many products which could directly or indirectly affect NK and LAK cells, including arachidonic acid metabolites, reactive oxygen metabolites, proteases, transferrin, IL-1, or tumor necrosis factor (6). Despite this, we were unable to detect a soluble mediator of LAK inhibition. Neither 24-h AM culture supernatants nor the use of porous Transwell membranes resulted in any suppression. Suppression occurred only when AMs were added directly into the LAK assay. These findings were supported by the addition of specific blocking agents. Neither indomethacin, nor catalase, nor SOD altered the inhibitory capacity of AMs. We also performed single-cell binding studies to determine if specific cell-to-cell interactions occurred between AMs and either the tumor targets or LAK effectors. LAK cells, as expected, demonstrated a high frequency of target cell binding with approximately 45% of the effector population binding either M14 or K562. When tested at 37°C both AMs and PBMs also demonstrated a high frequency of tumor binding which was nearly equal to that of LAK cells. This binding was, however, of equal magnitude for both targets and for both mononuclear phagocytes, making selective target binding an unlikely candidate for the mechanism of LAK inhibition. Interestingly, the binding interaction between LAK cells and either AMs, PBMs, or PBLs paralleled their inhibitory capacity. Less than 3% of LAK cells bound PBLs and minimal inhibition was seen when PBLs were added into the LAK assay. Approximately 7.5% of LAK effectors bound PBMs and PBMs were only weak inhibitors of LAK function. Finally, 17% of LAK cells bound AMs, and AMs were the most potent inhibitors of LAK activity. These findings raised the possibility that AMs were acting as alternate LAK targets and producing inhibition by cold target competition. Djeu and Blanchard (27) recently demonstrated that fresh PBMs are extremely poor LAK targets but that in vitro differentiation makes them 3 to 5 times more susceptible to lysis. We hypothesized that AMs may represent the equivalent of a "differentiated PBM" and therefore act as an alternate LAK target. We also speculated that this may represent a mechanism of pulmonary toxicity during IL-2/LAK therapy. Direct testing of this hypothesis, however, demonstrated no significant difference between AMs and fresh PBMs with respect to lysis by LAK cells. Both are 200 to 400 times more resistant to lysis than the standard M14 and K562 tumor targets used in this report. We cannot, however, rule out the possibility that AMs are recognized and bound as LAK targets but resistant to LAK-mediated lysis. We also cannot rule out the possibility that the greater binding between AMs and LAK cells, as compared to that between PBMs and LAK cells, is due to the larger size of the AM rather than a different functional capacity. In total, these results suggest that LAK inhibition requires direct cell contact, probably between the AM and LAK cell, and is independent of the release of soluble mediators. Under these conditions we were not surprised to find that AMs from both smokers and nonsmokers produced identical LAK inhibition. The same result was described by Weissman et al. (8) when they studied the effect of smoking on the inhibition of NK function. Both Weissman et al. and Weissler et al. (9) have hypothesized that it is the increased number of AMs in the lungs of smokers, rather than the function of the AMs, which results in a greater level of immunosuppression. Our finding of increased numbers of fully inhibitory AMs (3 to 7 times as many) in the BAL fluid of smokers is consistent with this hypothesis.

In summary, our results demonstrate that AMs obtained from the lungs of healthy subjects are extremely potent inhibitors of in vitro LAK function. This inhibitory capacity is not unique to the AM but is markedly amplified (8-fold) compared to that of the PBM. The effect of the AM is further amplified by its overwhelming presence in the lung where it constitutes 90 to 98% of the leukocyte population. The role of the AM in regulating LAK cell cytotoxicity in vivo remains to be tested, but the propensity of the lung to develop cancer, the poor response of lung cancer to current immunotherapy, the overwhelming presence of the AM, and the potential for interaction between transfused LAK cells and pulmonary macrophages all suggest that AMs may limit cytotoxic responses in the lung. This hypothesis deserves direct testing and may warrant consideration when designing adoptive immunotherapy protocols. We also demonstrated that smoking does not directly affect the inhibitory capacity of the AM but may result in a more suppressive environment due to the recruitment of 3 to 7 times as
many AMs into the lung. Finally, the actual mechanism of inhibition appears to require specific cell-to-cell contact, probably between the AM and the LAK cell, and appears to be independent of soluble mediators. The exact nature of this interaction between LAK cells and AMs remains to be discovered. One possibility is the presence of a receptor or cell surface signal which down-regulates LAK function. Preliminary work has demonstrated the ability of isolated AM membranes to mediate LAK inhibition and this may explain the residual inhibitory activity observed on formalin fixed AMs. It is also possible that AM:LAK binding allows the directed secretion of soluble mediators which were not detected by our assay methods.

ACKNOWLEDGMENTS

The authors appreciate the comments, support, and advice of Dr. Donald P. Tashkin and Dr. Hungyi Shau.

REFERENCES

Inhibition of Lymphokine-activated Killer Cell Function by Human Alveolar Macrophages

Michael D. Roth and Sidney H. Golub


Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/17/4690

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.