In Vitro Generation of Tumoricidal Properties in Human Alveolar Macrophages following Interaction with Endotoxin

Saburo Sone, Satoru Moriguchi, Eiji Shimizu, Fumitaka Ogushi, and Eiro Tsubura

ABSTRACT

Human alveolar macrophages (AM) obtained by bronchoalveolar lavage from healthy nonsmoking donors exhibited primarily low levels of cytolytic activity against allogeneic tumor target cells. These AM acquired enhanced capacity to kill tumor cells following a 24-hr incubation in vitro with endotoxin [lipopolysaccharide (LPS)]. Maximal tumoricidal activity of LPS-activated AM as measured by lysis of tumor target cells was obtained after incubation with tumor cells for 72 hr. LPS-activated AM lysed allogeneic tumor cell lines of different origins but did not affect normal, nonneoplastic cells.

We conclude that LPS induces human AM to become tumoricidal. This method should be useful in studies on therapeutic agents enhancing AM-mediated cytotoxicity in situ.

INTRODUCTION

There is increasing evidence from animal studies that cells of the macrophage-histiocyte series may play an important role in host defense against neoplasms. Murine macrophages can be activated to become cytotoxic against tumor cells by interaction in vitro with a variety of agents, such as lymphpokines, whole bacteria, and/or their products (1, 5, 7–10, 17, 18, 22, 28, 37). Under in vitro conditions, macrophages activated by these means can recognize and destroy neoplastic cells by a nonimmunological mechanism that requires cell-to-cell contact (3, 9, 10, 17).

The lung is a frequent site for the development of cancer metastasis, and the growth of metastatic tumor cells in lung is known to be influenced by the properties of both host tissues and host factors (11, 26). The integrity of AM may be important in the destruction of tumor cells reaching the lung. Similar to peritoneal macrophages, rodent AM can be rendered tumoricidal by treatment in vitro with various agents, such as lymphpokines and bacterial preparations such as LPS or muramyl dipeptide (32, 34) as well as Bacillus Calmette-Guérin and Corynebacterium parvum (25, 38). Moreover, in animal tumor systems, a close association was observed between the tumoricidal activity of the AM and the eradication of pulmonary metastasis (12). Whether or not these findings can be directly extrapolated to humans is questioned. In humans, peripheral blood monocytes have been shown to destroy tumor cells in vitro (16). Human monocytes also acquire enhanced cytotoxicity for human tumor target cells after treatment with lymphokines or LPS (4, 15). To date, however, little is known about the responsiveness of human AM, since macrophages other than monocytes have been difficult to obtain. However, with the recent widespread application of fiberoptic bronchoscopy, human AM are now available for such studies (20).

In an attempt to extend the studies previously limited to animal systems, we have obtained human AM from the lung of healthy nonsmoking volunteers by bronchoalveolar lavage. We found that human AM can respond in the same way as rodent AM to endotoxin (LPS) stimuli and can become cytostatic and cytolytic to human tumor target cells. Furthermore, we describe the kinetics and spectrum of the tumoricidal effects of human AM activated with LPS.

MATERIALS AND METHODS

Harvesting of Human AM. Appropriate informed consent was obtained from healthy nonsmoking donors before this study. Bronchoalveolar lavage was performed as described in detail elsewhere (24). Briefly, anesthesia of the oral cavity and the upper airway was accomplished with lidocaine spray (10 ml of 4% lidocaine suspension). An Olympus fiberoptic bronchoscope was passed p.o. and wedged into one of the segments of the right or left lobe. The lung was washed with 50 ml of 0.9% NaCl solution prewarmed at 37°C. The process was repeated 3 times by gentle suction with a 50-ml syringe. A total of 150 ml of 0.9% NaCl solution was instilled, of which approximately 60% was recovered.

Target Cell Lines. Human melanoma cell line A375 (14) was a gift from Dr. Isaiah J. Fidler (Frederick Cancer Research Facility, Frederick, Md.). The KB cell line (30) derived from a human epidermoid carcinoma was a gift from Dr. Kiyoshi Ishii (Biological Research Institute, Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan). HeLa cell line of human carcinoma of the cervix and Flow 7000 of human embryonic foreskin were obtained through Dai-Nippon Pharmaceutical Co., Inc., Tokyo, Japan, from Flow Laboratories, Inc., Rockville, Md. These cell lines were adapted for growth in vitro. Monolayer cultures were maintained on plastic in RPMI 1640 supplemented with 5% heat-inactivated FBS, penicillin G, and streptomycin at 37°C in a humidified atmosphere containing 5% CO2. For cytotoxicity assays, cultures were used in the exponential growth phase.

Preparation and Purification of Human AM Cultures. The lavaged cells were passed through sterilized gauze and washed twice. The total number of cells collected was determined with a hemocytometer (counting sample diluted with 2% acetic acid solution). The viability of nucleated cells as determined by trypan blue exclusion was greater than 95%. The cells were plated into wells of a Microtest II plate (Falcon Plastics, Oxnard, Calif.). Nonadherent cells (less than 10%) were removed 60 min after plating by washing with RPMI 1640. At that time, more than 99% of adherent cells were mononuclear and positive for esterase activity.

In Vitro AM Activation. In vitro AM activation was achieved by treating AM for 24 hr in RPMI 1640 supplemented with 5% FBS containing various concentrations of LPS (from Escherichia coli 0.55:135, obtained from Difco Laboratories, Detroit, Mich.). The AM...
cultures were washed thoroughly with fresh RPMI 1640 before addition of target cells.

**AM-mediated Cytotoxicity Assay in Vitro.** AM-mediated cytotoxicity was assessed by measuring release of radioactivity as described in detail previously (32, 34). Target cells in the exponential growth phase were incubated for 24 hr in 5% FBS-containing RPMI 1640 supplemented with 0.4 µCi [125I]iododeoxyuridine per ml (specific activity, 5 Ci/mg; Amersham International Ltd., Buckinghamshire, England). The cells were washed 2 times with warm RPMI 1640 to remove unbound radiolabel, harvested by brief trypsinization (0.25% Difco trypsin and 0.02% EDTA for 1 min at 37°), and resuspended in RPMI 1640 supplemented with 5% FBS; 1 x 10⁶ or 2 x 10⁶ cells were plated in the wells containing AM. The cultures were washed and refed with fresh RPMI 1640 supplemented with 5% FBS 16 hr after plating of target cells to remove nonadherent and dead target cells. After 56 hr, the AM:target cell cultures were washed twice with 0.01 M NaH₄PO₄, Na₂HPO₄, and adherent, presumably viable cells were lysed with 0.1 ml of 0.1 N NaOH. Samples of lysate were pooled, and radioactivity was measured in a gamma counter.

The percentage of cytotoxicity mediated by activated human AM was calculated as follows:

\[
\text{% of cytotoxicity} = \frac{\left( \frac{\text{cpm in target cells cultured with normal AM}}{\text{cpm in target cells cultured with test AM}} \right) - 1}{\text{cpm in target cells cultured with normal AM}} \times 100
\]

**Statistical Analysis.** The statistical significance of differences between test groups was analyzed by Student’s t test (2-tailed).

**RESULTS**

**Yield of Human AM.** One segment of the lung lobe was washed 3 times with 50 ml of sterilized 0.9% NaCl solution under the bronchoscopic examination. The lavage method used here consistently yielded about 18.1 ± 3.8 (S.D.) x 10⁶ cells/wedged segment of the lung lobe from 6 healthy non-smokers. The harvested cells consisted of 15.6 ± 4.1 x 10⁶ AM, 1.9 ± 1.3 x 10⁵ lymphocytes, and 0.4 ± 0.2 x 10⁶ neutrophils. Essentially all the adherent cells (>99%) were macrophages, as determined by their morphology and nonspecific esterase activity.

**Spontaneous Tumoricidal Activity of Human AM.** In the first set of experiments, we determined whether human AM from normal volunteers can kill tumor cells without any further stimulation (Table 1). Various numbers of AM were plated and incubated for 24 hr in RPMI 1640 supplemented with 5% FBS. The AM cultures were then washed and incubated for another 72 hr with 1 x 10⁴ [125I]iododeoxyuridine-labeled allogeneic A375 melanoma cells per well. The initial ratio of AM to tumor cells ranged in different test groups from 20:1 to 1:1. Decrease in the number of AM per well was associated with reduction in the natural cytotoxicity mediated by the AM. In the second set of experiments, AM samples were obtained from the lung of 10 different normal volunteers to test their spontaneous tumoricidal activity. Samples of 10⁵ AM preincubated for 24 hr in RPMI 1640 with 5% FBS were incubated for a further 72 hr with 1 x 10⁴ labeled A375 melanoma cells. The data shown in Table 1 demonstrate that their natural tumoricidal activities varied from 4 to 37%.

**In Vitro Augmentation of Tumoricidal Activity in AM by LPS.** To determine whether human AM are activated by LPS, we incubated 10⁵ AM for 24 hr with LPS (5 µg/ml) before addition of labeled A375 melanoma cells. Examination of the AM:tumor cell cultures after 4, 24, 48, and 72 hr showed that by 72 hr LPS-treated AM were highly and reproducibly cytotoxic to A375 cells (Chart 1). We next determined the dose of LPS necessary to achieve maximal AM activation (Table 2). Human AM were treated for 24 hr in RPMI 1640 with 5% FBS containing LPS at concentrations ranging from 0.05 ng/ml to 50 µg/ml before addition of 2 x 10⁴ labeled A375 melanoma cells. The data (Table 2) demonstrate that more than 5 ng of LPS per ml leads to significant AM-mediated cytotoxicity. However, in subsequent studies, maximal and reproducible levels of tumoricidal AM activity were demonstrated by treating them with LPS (5 µg/ml). In a parallel set of experiments (Chart 2), we varied the AM:tumor cell ratio by plating a constant number of tumoricidal AM activity were demonstrated by treating them with LPS (5 µg/ml). In a parallel set of experiments (Chart 2), we varied the AM:tumor cell ratio by plating a constant number of AM per well. The cytotoxicity mediated by LPS-activated AM was increased by introduction of more target cells into the well. Significant and reproducible levels of cytotoxicity were obtained by incubating 10⁵ AM with more than 5 x 10³ A375 melanoma cells.

**Kinetics of AM Activation by LPS.** As shown in Chart 3, a 24-hr incubation period was necessary for maximal activation of AM. Significant and reproducible levels of cytotoxicity were obtained by incubating 10⁵ AM with more than 5 x 10³ A375 melanoma cells.

**Table 1**  
Spontaneous tumoricidal activity of human AM

<table>
<thead>
<tr>
<th>Experimental set</th>
<th>No. of AM/1 x 10⁶ tumor cells</th>
<th>Radioactivity (cpm) in live A375 cells on Day 3</th>
<th>% of spontaneous cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 No AM, A375 cells alone</td>
<td>2923 ± 106³†</td>
<td>36⁴ ³</td>
</tr>
<tr>
<td></td>
<td>2 x 10⁵ AM (20:1)</td>
<td>1954 ± 42</td>
<td>16⁴ ³</td>
</tr>
<tr>
<td></td>
<td>1.5 x 10⁵ AM (10:1)</td>
<td>2400 ± 173</td>
<td>16⁴ ³</td>
</tr>
<tr>
<td></td>
<td>1 x 10⁶ AM (10:1)</td>
<td>2339 ± 138</td>
<td>20⁴ ³</td>
</tr>
<tr>
<td></td>
<td>0.2 x 10⁶ AM (2:1)</td>
<td>2802 ± 4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.1 x 10⁶ AM (1:1)</td>
<td>3648 ± 201</td>
<td>0</td>
</tr>
</tbody>
</table>

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<tr>
<th>% of spontaneous cytotoxicity at following no. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<tr>
<td>2</td>
</tr>
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</table>

³ Various numbers of human AM were incubated for 24 hr in RPMI 1640 supplemented with 5% FBS before addition of 1 x 10⁴ [125I]iododeoxyuridine-labeled A375 melanoma cells.
† Mean ± S.D. of triplicate cultures.
³ Numbers in parentheses, AM:tumor cell ratio.
⁴ Percentage of cytotoxicity as compared with tumor cells alone.
"p < 0.01.
'p < 0.05.
³ AM (10⁵) were incubated for 24 hr in RPMI 1640 with 5% FBS before addition of 1 x 10⁴ labeled A375 cells. The assays were terminated 72 hr later.
Activation of Human AM by LPS

Chart 1. In vitro cytotoxicity by human AM activated with LPS. AM (10⁶) were incubated for 24 hr with LPS (5 µg/ml) before addition of 1 x 10⁶ labeled A375 cells. The assays were terminated at the indicated times. Percentage of cytotoxicity was calculated as compared with untreated AM and tumor cells. Bars, S.D. of triplicate cultures. Summary of 3 independent experiments.

Table 2

<table>
<thead>
<tr>
<th>AM treatment</th>
<th>Human AM-mediated cytotoxicity (cpm)</th>
</tr>
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<tbody>
<tr>
<td>A375 cells alone</td>
<td>3004 ± 218b</td>
</tr>
<tr>
<td>Untreated AM</td>
<td>2807 ± 54</td>
</tr>
<tr>
<td>LPS 0.05 ng/ml</td>
<td>2714 ± 89</td>
</tr>
<tr>
<td>5 ng/ml</td>
<td>2157 ± 95 (23)c,d</td>
</tr>
<tr>
<td>0.5 µg/ml</td>
<td>1595 ± 5 (43)</td>
</tr>
<tr>
<td>5 µg/ml</td>
<td>1507 ± 124 (46)b</td>
</tr>
<tr>
<td>25 µg/ml</td>
<td>2154 ± 100 (23)c</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>2540 ± 100 (10)</td>
</tr>
</tbody>
</table>

a AM (10⁶) were plated for 1 hr and incubated for 24 hr with various concentrations of LPS. AM cultures were thoroughly washed before addition of 2 x 10⁶ labeled A375 melanoma cells.

b Mean ± S.D. of triplicate cultures; summary of 3 independent experiments.

c Percentage of cytotoxicity compared with untreated AM and tumor cells (p < 0.05).

d Numbers in parentheses, percentage.

* Percentage of cytotoxicity compared with untreated AM and tumor cells (p < 0.005).

24 hr with LPS (5 µg/ml) before addition of labeled target cells and incubations were continued for 72 hr. The next experiments were performed to examine the effect of pretreatment with lidocaine on ability of LPS to activate AM, since lidocaine is routinely used as a local anesthetic of the upper airways during bronchoscopic examination. AM were pretreated for 2 hr with RPMI 1640 containing lidocaine at concentrations of 0.2 to 2000 µg/ml. The AM were then thoroughly washed and were incubated for 22 hr with LPS (5 µg/ml) before addition of 2 x 10⁶ labeled A375 cells. Pretreatment of AM with lidocaine did not suppress their response to LPS at all (data not shown).

Spectrum of Cytotoxicity Mediated by LPS-activated AM. When AM were activated to become tumoricidal by treatment for 24 hr with LPS, they destroyed allogeneic A375, KB, and HeLa cell lines (Table 3). In contrast, neither untreated nor LPS-activated AM lysed normal, nonneoplastic human cells (Flow 7000).

DISCUSSION

In the present studies, we used human AM obtained by bronchoalveolar lavage of the lung of normal volunteers. Bronchoalveolar lavage during bronchoscopic examination is a useful way to obtain a large number of highly viable, purified mature macrophages. These AM showed low levels of cytolytic activity ranging from 4 to 37% after incubation for 24 hr in RPMI 1640 supplemented with 5% FBS. This was not the case in previous animal studies, which showed that murine AM require activation stimuli to show tumoricidal activity (12, 31-34). In human systems, the spontaneous cytotoxic activity of AM is controversial. Bordignon et al. (2) found that AM were not tumoricidal, although tumor cell proliferation was significantly inhibited by untreated AM obtained by lavage. In contrast, Lemarbre et al. (21) and Hisano et al. (19) reported that human AM exhibit primarily cytotoxic activity against tumor cells in vitro. These findings in the present study support the latter observations, since the incubation in vitro of cytolytic...
macrophages isolated from regressing tumors has been observed to result in reduction and/or loss of the activity (29). These discrepancies in natural cytotoxicity activity of human AM may be due to the different donors from whom AM were obtained, since these subjects may have been exposed to environmental stimuli previously, and also to the differences in the methods of cytotoxicity assay used and in the sensitivities of tumor cells used to macrophage-mediated cytotoxicity.

The data presented here demonstrate that human AM can respond directly to activation by endotoxin LPS. Cytotoxicity mediated by activated macrophages is thought to require cell-to-cell contact (3). The data shown in Chart 2 indicate that significant levels of in vitro cytotoxicity are correlated with the density of the AM:target monolayers, supporting the importance of cell-to-cell contact for AM-mediated tumor cell death. These results closely agree with our previous report (32) and those of others (7, 35) in animal models showing that macrophage-mediated cytotoxicity is dependent not on the macrophage:target cell ratio but on the population density of the monolayers per well used. LPS-activated human AM destroyed allogeneic tumor cells but were not cytotoxic to normal, non-neoplastic cells (Table 3). Thus, human activated AM can distinguish between normal and neoplastic cells in vitro. These data agree with our results obtained with rodent AM in a similar in vitro assay (31, 32, 34) and the recent observations of Lemarbre et al. (21) on human AM.

Macrophage activation by LPS has been studied extensively in animal systems (1, 7, 28, 37). Two activation signals have been described by Doe et al. (7, 8) who demonstrated that murine macrophages can be activated following the direct interaction with the lipid A moiety of protein-free LPS and a protein-rich material or lipid A-associated protein, entirely independent of B-lymphocytes. Since, in our present studies, essentially all the adherent cells obtained by lavage of the lung were mononuclear and gave a positive reaction for esterase, LPS activated human AM directly, not via interaction with lymphocytes. Maximal AM activation by LPS required incubation for 24 hr with LPS (5 μg/ml). We also found that incubation of AM for 24 hr with more than 25 μg of LPS per ml resulted in less cytotoxic activity, indicating the direct impairment of AM by LPS. This is consistent with a previous report by Davis et al. (6).

Recently, fiberoptic bronchoscopy has been routinely performed for the diagnosis of pulmonary diseases including neoplasms (20). Lidocaine is sprayed on the upper airway before and during bronchoscopic examination. There are some reports that local anesthetic agents suppress macrophage functions (13, 23). However, this was not the case in our studies when human AM were treated for 2 hr with various doses of lidocaine before AM activation with LPS. This finding is in accordance with that of Territo and Golde (36) on chemotaxis of human AM.

The question of whether human AM in lungs of patients with primary and/or metastatic tumors can respond to activation stimuli is of great importance. Pulmonary tumor growth has been shown to affect certain functions of AM (21, 27). Rhodes et al. (27) recently reported that human AM from lungs bearing primary cancer showed less ability to express Fc receptors than AM from normal subjects. Since, in animal models, AM of lungs bearing progressively growing pulmonary metastases have been found to be rendered tumoricidal by activation stimuli in vivo and in vitro (12, 33), further studies are required on the role of human AM in the presence of primary and/or metastatic pulmonary tumors.

In this study, we demonstrate that human AM can be activated in vitro by endotoxin (LPS) to destroy human allogeneic tumor cells. The approach used in this study may be valuable in future studies to evaluate effective therapeutic agents for enhancing tumor cytotoxicity activity of AM in situ.

REFERENCES

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Activation of Human AM by LPS


In Vitro Generation of Tumoricidal Properties in Human Alveolar Macrophages Following Interaction with Endotoxin
