Effect of Erythrocytes on Alveolar Macrophage Cytostatic Activity Induced by Bleomycin Lung Damage in Rats

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ABSTRACT

Bleomycin (BLM) has been successfully used to treat a number of human neoplasms. The main toxicity associated with BLM therapy is an acute pulmonary inflammation that can culminate in diffuse chronic fibrosis. The effect of BLM-induced pulmonary inflammation on the cytostatic activity of alveolar macrophages (AM) was investigated using AM obtained from rats that had been previously treated with BLM. Bronchoalveolar lavage fluid was collected at selected time intervals following a single fibrogenic dose of intratracheally administered BLM (3.6 mg/kg). AM obtained 12 to 72 h following intratracheal BLM (BLM-AM) caused cytostasis of murine leukemia L1210 cells in co-culture, whereas AM obtained from saline-treated controls were not cytostatic. These results indicate that the growth-inhibitory activity of the AM was related to the pulmonary inflammation. Cytostatic activity in control AM could be induced by in vitro exposure to lipopolysaccharide (5 µg). When AM were added to the AM-L1210 co-culture, the cytostatic activity of the BLM-AM was abrogated. The fact that chemical treatment of the BLM with sodium nitrite and potassium cyanide or N-ethylmaleimide did not alter the ability of the BLM to abrogate AM cytostatic activity suggests that the BLM is not acting as a scavenger of oxygen radicals. In contrast, the addition of FeSO₄ to the AM-L1210 co-culture mimicked the effect of BLM addition. Aconitase, an iron-sulfur-containing enzyme necessary for mitochondrial respiration, is decreased in L1210 cells that have been co-cultured with BLM-AM but not when the cultures also contain RBC. These results suggest that (a) pulmonary inflammation induces cytostatic activity in AM, (b) the alteration of iron homeostasis plays an important role in this cytostatic process, and (c) RBC can prevent this cytostatic activity.

INTRODUCTION

Inflammation is the hallmark of the acute pulmonary response to a variety of insults. Often this response results in the development of pulmonary fibrosis, which can severely compromise lung function (1). The fact that the lung is composed of more than 40 cell types (2) complicates studies directed at understanding the cellular processes involved in the resolution of acute inflammation. However, there appear to be specific cells, including the macrophage, the PMN, and the lymphocyte, that are instrumental in the inflammatory process.

A number of agents such as hyperbaric oxygen, amiodarone, paraquat, and BLM cause inflammatory lung disease (3–5). Indeed, the dose-limiting toxicity of BLM, a glycopeptide antibiotic used in the treatment of selected human neoplasms, is end-stage pulmonary fibrosis (6). Approximately 10–40% of patients treated with BLM will develop a dose-limiting pulmonary fibrosis, which has an overall mortality rate of 1%.

Although the exact mechanism of this toxicity is poorly understood, it is known that BLM, whether administered i.v. or i.t. as in many experimental protocols, causes an alveolitis that precedes fibrosis (6, 7).

We, as well as others, have shown that when BLM is administered i.t. there are two distinct phases of the disease (8, 9). Initially, there is an acute inflammation characterized by an influx of inflammatory cells and selected proteins into the alveolar space. Subsequently, there is a period of more chronic disease, during which the acute inflammation subsides and the laying down of fibrotic tissue begins. The fact that the disease process continues for the next 14–28 days when the drug has been cleared from the lungs suggests that other mediators are involved.

Since the AM represents greater than 95% of the cells present in the alveolar space (10) and the macrophage has been shown to have a variety of functions during an inflammatory response (11–13), it would seem probable that the AM plays a prominent role in the initial phase of this disease process. We have presented data showing that AM taken from BLM-treated rats spontaneously release IL-1 for 18 to 72 h following i.t. BLM administration. Further, we have shown that the release of IL-1 was not due solely to the direct effect of BLM on the AM (14). These observations have subsequently been confirmed and expanded to include other monokines released by the AM following i.t. BLM administration to rats or hamsters (15, 16). Thus, it appears that acute BLM-induced lung damage results in a rapid activation of AM, yielding a release of a number of monokines. Using i.t. administered BLM as a model of acute pulmonary inflammation, we have further investigated the effect of acute lung damage on AM function.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories, Canada. The animals were maintained on a 12-h dark, 12-h light cycle in a temperature- and humidity-controlled environment. They were housed, in facilities approved by the Association for Accreditation of Laboratory Animal Care at the University of Vermont, for a minimum of 1 week in cages covered with filter tops. Purina Rat Chow and water were available ad libitum.

Cells. Murine leukemia L1210 cells were routinely carried in antibiotic-free McCoy's 5A medium (GIBCO Laboratories) supplemented with 5% donor horse serum (Hazleton Biologics). Throughout the experiments, medium was further fortified with antibiotic-antimycotic solution (Sigma Chemical Co.), containing 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 µg/ml amphotericin B. Cells were negative for Mycoplasma (Cell Shipper DNA fluorochrome stain; Bionique Laboratories).

Induction of Pulmonary Inflammation and Collection of AM. Male Sprague-Dawley rats (200–250 g) received a fibrogenic dose (17) of BLM (Benloxxane; Bristol-Myers Company), 3.6 mg/kg given i.t. under a light anesthetic (chloral hydrate, 0.36 m, i.p.). Control animals received a comparable volume of sterile 0.9% NaCl. At selected times thereafter, animals were sacrificed with a pneumothorax following i.p. sodium pentobarbital (Anathy Products Co.). The lungs were perfused with 5 ml of sterile NaCl via the pulmonary artery, removed, and lavaged with 50 ml of ice-cold (15°C) modified PBS (2.5 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 8.1 mM NaHPO₄, pH 7.4). BAL fluids were collected in 10-ml syringes.
cells were centrifuged, resuspended in medium, and layered onto a discontinuous Percoll (Pharmacia) gradient (densities: >1.080, 1.064, and 1.054 g/ml). Gradients were centrifuged (700 × g) for 30 min at 4°C. Cell populations of three densities were collected and washed 3 times with PBS before being resuspended in a final volume of 1 ml of medium.

Assay for Macrophage-mediated Cytostasis. L1210 and AM were co-cultured in 96-well microtiter plates (Corning), with each well containing 50,000 effector cells and 5,000 L1210 target cells in 0.2 ml of medium. After 72 h the co-cultures were pulsed with 0.5 μCi of [3H]-thymidine (specific activity, 5 μCi; ICN Radiochemicals) for 2 h and harvested onto glass filters using a PHD cell harvester (Cambridge Technology, Inc.). To select co-cultures, various components were added (RBC, RBC lysate, or FeSO₄). Additionally, in some experiments RBC were chemically modified with N-ethylmaleimide or sodium nitrite and potassium cyanide, as described by Sweder van Asbeck et al. (18). Briefly, RBC were obtained by cardiac puncture from animals upon sacrifice and suspended in Ca²⁺- and Mg²⁺-free PBS (1 ml blood in 10 ml PBS). The RBC suspension was centrifuged and the cell pellet was resuspended in PBS, which was layered onto 60% Percoll (Percoll diluted with 0.15 m NaCl) and centrifuged (700 × g) for 30 min at 4°C. This purified RBC pellet was washed 3 times with PBS and resuspended in 1 ml of saline. Two hundred and fifty μl of RBC suspension were treated chemically as described (18). Following treatment, RBC were washed 5 times before being added to co-cultures.

Aconitase Assay. Co-cultures were established by adhering 5 × 10⁶ macrophages for 1 h in each well of a 24-well tissue culture plate. Any nonadherent cells were removed and 5 × 10⁶ L1210 cells were added. After 15 h, L1210 cells were removed and assayed for aconitase activity as described by Drapier and Hibbs (19). Briefly, L1210 cells were removed from the co-culture, centrifuged (180 × g) for 10 min at 4°C, washed once in 10 ml of PBS, and resuspended in 40 ml of PBS and 10 μl of 10% digitonin (Sigma). The cells were then centrifuged as above, resuspended in 250 μl of a sucrose solution [0.25 M sucrose, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 10 mM MgCl₂] and lysed with 250 μl of 0.4% Triton X-100 (in 0.15 M NaCl with 30 mM triethanolamine-HCl, pH 7.2). The lysate was centrifuged (5000 × g) for 10 min at 4°C and the supernatant fluid was immediately assayed for aconitase activity by following the disappearance of cis-aconitate spectrophotometrically, at a wavelength of 240 nm. The reaction mixture consisted of 0.5 ml of lysate, 100 μl of 1 mM cis-aconitate, and 50 μl of 30 mM triethanolamine buffer with 0.02% bovine serum albumin.

RESULTS

Cytostatic Activity of AM. Initially, excised rat lungs were lavaged 24 h after i.t. BLM administration, a time when AM are actively secreting IL-1 and PMN have entered the alveolar space (14, 15). The heterogeneous BAL cell population was assessed for cytostatic activity against L1210 cells in vitro (Table 1). L1210 cell growth was inhibited by BLM-AM but not by C-AM. To determine what the direct effect of BLM on the AM was, C-AM were incubated with several nontoxic doses of BLM in vitro for 24 h and the supernatant was removed and replaced with fresh medium prior to being co-cultured with L1210 cells. As shown in Table 1, C-AM exposed to 0.1 μg BLM in vitro were not cytostatic, but those exposed to 1 μg had a significant effect on uptake of thymidine by L1210 cells. A well documented feature of macrophage activation following in vitro stimulation with agents such as LPS is the acquisition of cytotoxic or cytostatic activity (20). Cytostatic activity could be induced in C-AM by the addition of LPS (5 μg) to the co-culture of C-AM with L1210 cells.

Inspection of these data reveals a large variability in cytostatic activity among BLM-AM. This could have been due to individual animal differences in response to the toxic effects of i.t. BLM or to the fact that several cell types are present in the BAL which might have modified the cytostatic activity of the BLM-AM. To test this, BAL was layered onto a discontinuous Percoll gradient and three cell populations were isolated (Table 2). All three cell populations were tested for cytostatic activity against L1210 cells and both BLM-AM cell populations were routinely cytostatic (Fig. 1). In contrast, neither the PMN pellet obtained from the Percoll gradient nor any cell type isolated from the BAL of control rats had cytostatic activity against L1210 cells. Consequently, all subsequent studies were performed using the Percoll-purified AM.

The mechanism of growth inhibition of tumor cells by AM

Table 2 Purification of BAL on Percoll density gradients

<table>
<thead>
<tr>
<th>Layer</th>
<th>Specific gravity (g/ml)</th>
<th>AM (%)</th>
<th>PMN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.064–1.047</td>
<td>93</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>1.074–1.064</td>
<td>83</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>&gt;1.080</td>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>BAL</td>
<td>58</td>
<td>42</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Cytostatic activity of BAL populations

<table>
<thead>
<tr>
<th>Effector cell population</th>
<th>[3H]-Thymidine incorporation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control AM</td>
<td>100 ± 0.3</td>
</tr>
<tr>
<td>Control AM + BLM (1.0 μg)</td>
<td>34 ± 10*</td>
</tr>
<tr>
<td>Control AM + BLM (0.1 μg)</td>
<td>78 ± 11</td>
</tr>
<tr>
<td>Control AM + LPS (5 μg)</td>
<td>3 ± 2.6*</td>
</tr>
<tr>
<td>BLM-AM</td>
<td>61 ± 44*</td>
</tr>
</tbody>
</table>

* P < 0.05.

Fig. 1. Cytostatic activity of BAL cell populations separated by Percoll density gradients. Co-cultures were established in 96-well microtiter plates, each well containing 50,000 effector cells and 5,000 L1210 cells in 0.2 ml of medium. After 72 h the co-cultures were pulsed with [3H]-thymidine (0.5 μCi) for 2 h and harvested. Results are expressed as the percentage of thymidine incorporated by co-cultures compared to controls (wells containing L1210 alone; typical cpm were 20,000). Error bars, 1 SD (n = 20 for each cell population). Results obtained for control and treated animals were analyzed by Student's t test. A P < 0.05 was considered significant. For saline-treated controls, both unseparated BAL and Percoll-purified cell populations routinely contained >95% AM and <5% PMN. The cell distributions for the various populations taken from BLM-treated animals are given in Table 2. Cultures containing macrophages alone do not incorporate a significant amount of thymidine (cpm < 100). In unseparated BAL, 1.064–1.047 g/ml; 1.074–1.064 g/ml; >1.080 g/ml; * P < 0.05.
has not been elucidated. A number of macrophage products, including IL-1 and tumor necrosis factor, have been reported to inhibit cell growth (21, 22). To test whether an AM-secreted product was the cytostatic agent, AM obtained 24 h after i.t. BLM administration (the time of peak IL-1 secretion by BLM-AM) were incubated for 18 h in vitro and the AM-conditioned medium was assayed for cytostatic activity. The fact that no cytostatic activity was detected in the AM-conditioned medium (data not shown) suggests that tumor growth is not restricted by monokines secreted by BLM-AM. This does not completely rule out a role for IL-1, since Inamura et al. (23) have recently reported that membrane-bound IL-1 had cytostatic activity, nor does it rule out the possibility of some short lived macrophage product.

Time Course of BLM-AM Cytostatic Activity. The production of monokines following in vivo activation by BLM-induced lung damage appears to be very dynamic, with different proteins being released at specific times following BLM administration (24). To determine whether AM cytostatic activity also occurs during a defined period of time following BLM administration, rats were given BLM, and AM were obtained at selected times thereafter (Fig. 2). AM cytostatic activity was noted as early as 12 h after BLM treatment but had returned to control levels by day 7. Inasmuch as the waning of AM cytostatic activity is temporally associated with the major influx of lymphocytes (Fig. 3), it is possible that the recruited lymphocytes are modulating the AM response to BLM-induced lung damage. Thus, as with the production of monokines, AM cytostatic activity appears to be a highly regulated response to BLM-induced lung damage.

RBC Modulation of BLM-AM Cytostatic Activity. As stated earlier, prior to Percoll separation the cytostatic activity of BAL cells was variable. To determine if another cell population in the BAL inhibited AM cytostatic activity, purified BLM-AM cell populations were co-cultured with purified PMN from the Percoll pellet or with RBC, also purified over a Percoll gradient. This cell mixture was assayed for cytostatic activity against L1210 cells. Only those cultures containing RBC were inhibitory in this system and the extent of inhibition was directly related to the number of RBC added to the culture (Fig. 4). In no study did the PMN have any effect on BLM-AM cytostatic activity, nor did the PMN have any such activity of their own. Further, the cytostatic activity of C-AM activated with LPS or 1 μg BLM in vitro was not abrogated by the addition of RBC to the co-culture (data not shown). These results suggest that the mechanism(s) activated by these agents is different from that which results when pulmonary inflammation is induced by BLM.

Activated AM have been shown to release a number of toxic oxygen species, any one of which has the potential to affect tumor cell growth (25, 26). Therefore, the protective activity of the RBC may reside in the fact that the RBC have the capacity to dissipate these toxic moieties through the abundant heme or glutathione constituents present in the RBC cytosol. To test this, RBC were treated with sodium nitrite, to induce methemoglobin, and cyanide, to stabilize the heme as the cyanomethemoglobin form and to inhibit endogenous RBC catalase.

Fig. 2. Cytostatic activity of AM at selected time intervals following i.t. BLM. BAL was collected at various time intervals following i.t. BLM and AM were isolated on Percoll density gradients. AM (1.064–1.074 g/ml) were co-cultured with L1210 cells and harvested as described in Fig. 1. Results are expressed as the percentage of thymidine (TdR) incorporated by co-cultures compared with controls (cells containing L1210 alone; typical cpm were 20,000). Error bars, 1 SD (n = 10 for each condition). Data were analyzed by analysis of variance. A P < 0.05 was considered significant. * P < 0.05.

Fig. 3. Cell distribution in BAL at selected time intervals following i.t. BLM. BAL cells were collected at selected time intervals, as described in “Materials and Methods.” Cytospin preparations were made and stained with Wright’s stain for differential cell counts. LYMPH, lymphocytes.

Fig. 4. Modulation of BLM-AM cytostatic activity. Co-cultures were established as described in Fig. 1, using AM (1.064–1.074 g/ml) collected 24 h post i.t. BLM. To selected wells, various components were added in the following concentrations: PMN, 5 × 10⁶; RBC lysate, 1.0 × 10⁸ (prepared by sonication of RBC); whole RBC, concentrations shown in the figure. Results are expressed as the percentage of thymidine (TdR) incorporated by co-cultures compared with controls (wells containing L1210 alone; typical cpm were 20,000). Error bars, 1 SD (n = 10 for each condition). Data were analyzed by analysis of variance. A P < 0.05 was considered significant. * P < 0.05.
Other RBC were treated with N-ethylmaleimide to deplete cells of reduced glutathione, as previously reported by Sweeder van Asbeck et al. (18). Neither chemical treatment had any effect on the protective capability of the RBC (Table 3), suggesting that the protective mechanism of the RBC in the BLM-AM-L1210 co-culture is not one of oxygen free radical scavenging. Whether the RBC were simply impeding target-effector cell contact through some physical means was addressed by adding RBC lysate to the cytostasis assay system. As with the intact RBC, the lysed RBC, albeit at a 4-fold higher RBC concentration, could also prevent AM-mediated cytostasis (Fig. 4).

Rescue of L1210 Cells by FeSO4. In a series of reports, Hibbs and his co-workers (19, 27) have provided convincing evidence that the cytostatic activity of the in vitro-activated macrophage results, at least in part, from sequestration of iron from the L1210 tumor cell. Iron appears to be removed from specific sites within the cell, such as Fe-S complexes, resulting in the inhibition of the electron transport system. Since the primary metal ion present in the RBC is iron, it seemed possible that the inhibition of AM cytostasis by the RBC could be through iron replacement by the RBC. To test this, BLM-AM were co-cultured with L1210 cells in the presence of 0, 50, or 500 µM FeSO4. As summarized in Table 3, FeSO4 had a marked inhibitory effect on the cytostatic activity of BLM-AM, not dissimilar from that observed with RBC.

Aconitase Activity. The iron-sulfur-containing enzyme aconitase has been shown by Hibbs and co-workers (19) to be decreased in L1210 cells exposed to LPS-stimulated peritoneal macrophages. This event coincides with the kinetics of inhibition of DNA synthesis (19). To determine whether BLM-AM had a similar effect on aconitase activity in L1210 cells, co-cultures were established and aconitase activity was measured. As depicted in Fig. 5, aconitase activity is significantly decreased in L1210 cells when co-cultured with BLM-AM but not C-AM, supporting the hypothesis that an early site of damage in the target cell is the iron-containing enzyme aconitase. Further, when RBC were added to the BLM-AM-L1210 co-culture, aconitase activity was significantly greater than the co-cultures which lacked RBC.

DISCUSSION

Our results demonstrate that BLM-induced acute pulmonary inflammation results in the activation of AM cytostatic activity. We have demonstrated that AM exhibit cytostatic activity as early as 12 h after i.t. BLM and remain activated for at least 72 h. Interestingly, this time frame corresponds to the inflammatory cell influx seen in the BAL. The activated AM may be responsible for recruitment of PMN into the area, as well as the subsequent tissue damage which continues long after BLM has been cleared from the area. This activation is relatively rapid in onset and short lived in nature. The cytostatic activity in vitro requires co-culturing of the target and effector cells, suggesting the necessity of intimate target-effector cell contact. Further, our data clearly demonstrate that cytostasis can be abrogated by the addition of iron to the assay system through the addition of intact RBC, RBC lysate, or FeSO4. Although not conclusive, these data lend credence to the hypothesis that AM cytostatic activity results from altered iron stores in the tumor cell. How the cytostatic activity of the AM is inhibited by the RBC is at present unknown, but it does appear to be related in some way to iron metabolism by either the target cell or effector cell.

The fact that 0.1 µg BLM, a dose previously reported to generate maximal activation of macrophages (28), failed to activate the C-AM in culture suggests that BLM is not solely responsible for AM activation in this model. Rather, BLM interaction with lung tissue and alveolar cells results in an AM

![Fig. 5. Modulation of aconitase activity in L1210 cells. BAL was collected 24 h post i.t. BLM and AM (1.064-1.047 g/ml) were isolated on Percoll gradients. Five million AM were plated into 24-well plates and allowed to adhere at 37°C in 10% CO2, for 2 h. Nonadherent cells were removed and 500,000 L1210 cells were added to each well. After 18-24 h, the nonadherent cells (>85% L1210) were harvested and assayed for aconitase activity as described in "Materials and Methods." A, C-AM + L1210 (A). C-AM treated with LPS + L1210 (B). C-AM treated with LPS + L1210 RBC (C). B, BLM-AM + L1210 (4). BLM-AM plus L1210 plus RBC (B). L1210 alone (C) (n = 8 for each co-culture). Macrophages taken from treated or control animals do not have any significant aconitase activity (data not shown).]
which is cytostatic to L1210 cells when taken from the lungs and put into a co-culture. At the higher dose of 1 mg BLM, there is a significant decrease in uptake of thymidine by L1210 cells, which is not affected by the addition of RBC to the co-culture. It is possible that direct addition of BLM to macrophages results in activation of a cytotoxic, rather than cytostatic, mechanism. Alternatively, at higher doses of in vitro drug, it is possible that the macrophage becomes saturated with drug and acts as a vesicle for direct delivery of BLM to the L1210 cell. In either case, our results suggest a novel mechanism of antitumor activity of BLM. These possibilities are currently under investigation in our laboratory.

Investigations by others reveal that insufflation of RBC to rats prior to exposure to 95% oxygen results in a significant decrease in the toxicity of the gas (18). These authors present data which suggest that glutathione, a prominent RBC protein, protects lung cells by scavenging toxic oxygen moieties. They therefore proposed that hemorrhage into the site of inflammation may play an important role in modulating the extent of damage incurred by the inflammation. In our system, the mechanism of RBC protection does not appear to be one of oxygen radical scavenging by the major RBC proteins. Nonetheless, our results support the hypothesis that RBC may alter the extent of pulmonary damage associated with this disease process by modulating AM activity in the lung. Further, it is possible that the presence of RBC within a tumor, which occurs following tumor neovascularization and in hemorrhagic areas of tumor necrosis, could significantly impair the growth inhibitory capacity of the tissue macrophage. Thus, a better understanding of the mechanism of activation and cytostatic activity of the AM will be valuable in understanding the role of the macrophage in inflammatory disease states. Similarly, further studies on the RBC-AM interaction may provide important information concerning tumor growth control by the host macrophage in the intact animal.

A number of investigators have recently presented data which suggest that activated macrophages are able to secrete nitric oxides through an L-arginine-dependent mechanism (29-31). As shown by Hibbs et al. (29), this effector mechanism is capable of causing inhibition of DNA synthesis as well as mitochondrial respiration. They further demonstrate that acitoxitae activity is also reduced. Stuehr and Nathan (32) have confirmed this observation using macrophages activated with γ interferon and LPS in co-culture with L1210 cells. In our model of in vivo inflammation, the macrophages taken from bleomycin-treated animals may be spontaneously releasing nitric oxides into the culture medium. These nitrogen intermediates may in turn be causing oxidative damage to the iron-sulfur-containing enzymes of the mitochondria in the target cell. Further, the RBC may be acting as a scavenger of these toxic moieties both in vitro and in vivo.

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