Role of Reactive Nitrogen Intermediate Production in Alveolar Macrophage-mediated Cytostatic Activity Induced by Bleomycin Lung Damage in Rats

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ABSTRACT

Bleomycin (BLM) is a useful anticancer agent sometimes associated with a diffuse pulmonary inflammation and fibrosis. Using an intratracheal model of BLM-induced pulmonary damage, we have further investigated alveolar macrophage (AM) activation following intratracheal BLM. From rats that had been treated with either a single, fibrogenic, intratracheal dose of BLM (BLM-AM) or a comparable volume of saline (C-AM), bronchoalveolar lavage fluid was collected, and AM were isolated using Percoll gradient centrifugation. Using a spectrophotometric assay, production of nitrates by AM was measured. C-AM released low levels of nitrates, whereas BLM-AM as well as C-AM activated in vitro with lipopolysaccharide released significant amounts of nitrates. The addition of \( N^\delta \)-monomethyl arginine, a substrate-specific inhibitor of the \( L \)-arginine-dependent effector mechanism in activated macrophages, reduced the amount of measurable nitrates released from both BLM-AM and activated C-AM. Similar results were observed when 12 \( \times 10^6 \) RBC were added to the cocultures. In the presence of \( N^\delta \)-monomethyl arginine, BLM-AM had no effect on two consequences of BLM-AM-induced cytostatic activity, DNA synthesis inhibition and aconitase activity reduction in the L1210 target cell. These results suggest that reactive nitrogen intermediates measured as nitrates are important moieties in our \( in vivo \) model of macrophage activation. Further, the identification of this effector molecule presents possibilities for therapeutic and biochemical manipulations.

INTRODUCTION

BLM is useful in the treatment of several human neoplasms (1). The main toxicity associated with BLM therapy is a dose-limiting pulmonary fibrosis which occurs in 10 to 40% of all patients treated and is fatal in 1% of all cases (2, 3). Whether the drug is given i.v. as in the clinical setting or i.t. as in many experimental models, the resulting biochemical composition and histological appearance of the lungs are similar (3, 4).

The predictability of the disease onset and the low dose of drug required to induce it have prompted numerous investigators to use this model for the study of BLM lung disease. The pulmonary response can be separated into two distinct phases (5, 6). (a) An acute inflammatory response occurs during which time inflammatory cells and edema are present in the alveolar space. (b) A chronic phase develops characterized by resolution of the acute inflammation and deposition of fibrotic tissue.

Although BLM is cleared from the lungs soon after it is given i.t. (7), the disease process progresses and culminates in a focal, chronic fibrosis 21 to 28 days after administration. Since the AM comprises greater than 95% of the cells normally present in the alveolar space (8) and functions in a variety of ways during an inflammatory response (9–11), it seems reasonable that this cell may play a pivotal role in modulating this disease process. Indeed, macrophages taken from animals treated with BLM i.t. have been shown to secrete inflammatory mediators, such as interleukin 1 (12–14), and growth promotion factors, such as fibroblast growth factor (15, 16).

A recent series of reports has established that AM activated \( in vitro \) with various cytokines, such as \( \gamma \)-interferon and tumor necrosis factor, or LPS secrete RNI through an \( L \)-arginine-dependent mechanism (17–20). Others have demonstrated that activation of an \( L \)-arginine-dependent mechanism in endothelial cells results in production of EDRF which has characteristics similar to NO (21, 22). The vasorelaxation caused by EDRF as well as the respiratory inhibition in target cells caused by NO release from activated macrophages is inhibitable by ferrous hemeproteins (20, 23).

We, as well as others (24, 25), have reported that RBC are capable of modulating macrophage-tumor cell interactions. Previously, we demonstrated that BLM-AM were cytostatic to murine leukemia L1210 cells. These AM not only inhibited DNA synthesis but also caused a significant reduction in the level of aconitase activity in the damaged target cell. Further, the inhibition of both DNA synthesis and aconitase activity was completely abrogated by the addition of RBC to the coculture.

We now report that AM activated \( in vivo \) by BLM-induced lung damage spontaneously secrete nitrates. Further, we demonstrate that this secretion can be blocked by a substrate-specific analogue of the \( L \)-arginine-dependent effector mechanism, NMA. In addition, the presence of RBC in the coculture causes a significant reduction in the amount of measurable nitrates by a mechanism which is not yet defined. Further, the ability of BLM-AM to cause either DNA synthesis inhibition or aconitase activity reduction in target cells is lost in the presence of NMA.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were obtained from the NIH, Bethesda, MD. 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 Animal Accreditation of Laboratory Animal Care at the University of Vermont, in cages covered with filter tops for a minimum of 1 wk prior to the study. Purina rat chow and water were available ad libitum.

Cells. Murine leukemia L1210 cells were routinely grown at 37°C in a humidified environment of 90% air and 10% CO\(_2\) in antibiotic-free McCoy's 5A Medium supplemented with 5% DHS. For experimental purposes, MEM with 5% DHS, 1% glutamine, 1% MEM essential vitamins, and an antibiotic-antimycotic containing 100 units/ml of penicillin, 0.1 mg/ml of streptomycin, and 0.25 \( \mu \)g/ml of amphotericin B was used. To avoid potential interference with the spectrophotometric assay for nitrates described below, this medium did not contain phenol...
red. Both media were obtained from Gibco, Grand Island, NY; the DHS was purchased from JRH Biosciences, Lenexa, KS; and the glutamine, vitamins, and antibiotics were from Sigma Chemical Company, St. Louis, MO. Cells were negative for Mycoplasma (Cell Shipper DNA fluorochrome stain; Bionique Laboratories).

Induction of Pulmonary Inflammation and Collection of AM and RBC. Rats, 200 to 250 g, received a single fibrogenic dose of BLM (Benoxane; a generous gift from Bristol-Myers Co.), 3.6 mg/kg (15) given i.t. while under light anesthesia induced by i.p. chloral hydrate. Control animals received a comparable volume of 0.9% saline (NaCl) solution i.t. Twenty-four h later, animals were sacrificed with a pneumothorax following i.p. sodium pentobarbital (Anothy Products Co.). The lungs were removed and lavaged with a total of 50 ml of ice-cold Ca2+- and Mg2+-free PBS (2.5 mM KCl:1.5 mM KH2PO4:137 mM NaCl:8.1 mM Na2HPO4, pH 7.4). Bronchoalveolar lavage fluid cells were centrifuged, resuspended in medium, and layered onto 60% Percoll (Pharmacia; density, 1.064 g/ml), which was centrifuged (700 x g) for 30 min at 4°C. The mononuclear cells were collected at the medium-Percoll interface and washed 3 times with cold PBS. The final cell pellet was resuspended in medium, and a cytopsin preparation was made and stained with Wright's stain (Diff Quik; VWR) for a differential cell determination. This cell population was routinely >90% macrophages with the remaining cells being polymorphonuclear cells. RBC were obtained by cardiac puncture from the rat upon sacrifice and were suspended in PBS (1 ml of blood in 10 ml of PBS). The RBC suspension was centrifuged, and the cell pellet was resuspended in 5 ml of PBS and layered onto 5 ml of 60% Percoll. After centrifugation (700 x g) for 30 min at 4°C, the purified RBC pellet was then washed 3 times with cold PBS and resuspended in medium.

Assay for Macrophage-mediated Cytostasis. L1210 and AM were cocultured in 96-well microtiter plates (Corning), with each well containing 3 x 10⁴ effector cells and 3 x 10⁵ L1210 target cells in 200 μl of MEM. After 24 h, 100 μl of supernatant were removed for the nitrite assay. Fresh medium, 100 μl, was returned to the cocultures which were then pulsed with 0.5 μCi of [3H]thymidine (specific activity, 6.7 Ci/mmol; ICN Radiochemicals) for 2 h before being harvested onto glass filters with a PHD harvester (Cambridge Technology, Inc.). In some experiments, 250 μM final concentration NMA (Sigma Chemical Co.), 12 x 10⁴, or medium was added to the cocultures.

Acconitase Assay. Concurrently with the cytostasis assay, cocultures were established by adhering 5 x 10⁴ AM for 1 h in 24-well tissue culture plates (Corning). Any nonadherent cells were removed by gently washing the wells with PBS, and 5 x 10⁴ L1210 cells were added in a total volume of 1 ml. Twenty-four h later L1210 cells were removed and assayed for acconitase as previously described (24, 26). To selected wells NMA, 250 μM final concentration, or 40 x 10⁴ RBC were added. Acconitase activity was determined by measuring the disappearance of cis-aconitate (Sigma Chemical Co.) spectrophotometrically at a wavelength of 240 nm. Results are expressed as nmol of aconitase/min/10⁶ cells.

Nitrite Assay. From the cocultures established for the cytostasis assay, 100 μl of supernatant were taken and mixed with an equal volume of Greiss reagent (1% sulfanilamide:0.1% naphthylethylenediamine dihydrochloride:2.5% H3PO4), incubated for 10 min at room temperature, and read spectrophotometrically at 550 nm as previously described (18). Sodium nitrite, 1 μM, was serially diluted and assayed simultaneously to obtain a standard curve. Results are expressed as nmol of nitrite/3 x 10⁵ AM. Chemicals were purchased from Sigma Chemical Company.

Statistical Analysis. Data were analyzed for significance by analysis of variance or Student's t test using a software package developed at the University of Vermont. A P < 0.05 was considered to be statistically significant.

**RESULTS**

Release of Nitrites. In a previous study (24), we established that AM had become cytostatic within 24 h following an i.t. dose of BLM (3.6 mg/kg). Several investigators have shown that macrophages activated in vitro release nitrites into the tissue culture medium (17-20, 23). To determine if BLM-AM were producing nitrites during the time when BLM-AM were cytostatic, we cocultured AM and L1210 cells for 24 h and assayed the medium for nitrites. As depicted in Fig. 1, there is significant production of nitrites by the BLM-AM, and they continue to release nitrites up to 48 h (data not shown) in tissue culture. While C-AM release only a small amount of nitrites, they can be activated in vitro to release nitrites by the addition of LPS (Fig. 1).

Modulation of Nitrite Production. In order to determine if the nitrites were being produced through the specific L-arginine-dependent effector mechanism described by Hibbs et al. (17), NMA was added to the AM cultures. The data in Table 1 demonstrate that, whether AM were activated in vivo or in vitro, nitrite production was substantially inhibited by NMA.

We have previously shown that RBC prevented the cytostatic activity of BLM-AM (24). To establish if the RBC also prevented the cytostatic activity of the AM, we cultured the AM with and without BLM (3.6 mg/kg). Several investigators have shown that AM had become cytostatic within 24 h following an i.t. dose of BLM (3.6 mg/kg). While C-AM release only a small amount of nitrites, they can be activated in vitro to release nitrites by the addition of LPS (Fig. 1).

**Modulation of Nitrite Production with NMA and RBC**

Animals were given an i.t. dose of 0.9% NaCl solution or 3.6 mg/kg of BLM and sacrificed 24 h later. Cells were collected and purified as described in "Materials and Methods." Cocultures were established containing 3 x 10⁵ AM and 3 x 10⁵ L1210 cells and incubated for 18 to 24 h at 37°C in 10% CO2. Following incubation, the supernatant was assayed for the presence of nitrites as described in "Materials and Methods." In selected experiments, macrophages taken from control animals were activated in vitro for 24 h with LPS (5 μg) prior to the addition of L1210 cells and the 18-24 h coculture. A P < 0.05 was significant. *, P < 0.05. Columns, mean; bars, SEM (n = 23 for each condition).

**Table 1 Modulation of nitrite production with NMA and RBC**

Animals were given an i.t. dose of 0.9% NaCl solution or 3.6 mg/kg of BLM and sacrificed 24 h later. Cells were collected and purified as described in "Materials and Methods." Cocultures were established as described in Fig. 1. To selected cocultures 12 x 10⁴ RBC or 250 μM NMA was added. In selected experiments, macrophages taken from control animals were activated in vitro for 24 h with LPS (5 μg) prior to the addition of L1210 cells and the indicated modifiers. Results are expressed as nmol of nitrites produced per 3 x 10⁵ macrophages.

<table>
<thead>
<tr>
<th>Macrophage source</th>
<th>Modifier added</th>
<th>Nitrite (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-AM</td>
<td></td>
<td>0.51 ± 0.21*</td>
</tr>
<tr>
<td>C-AM</td>
<td>12 x 10⁴ RBC</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>C-AM</td>
<td>250 μM NMA</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>BLM-AM</td>
<td></td>
<td>1.80 ± 0.28*</td>
</tr>
<tr>
<td>BLM-AM</td>
<td>12 x 10⁴ RBC</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>BLM-AM</td>
<td>250 μM NMA</td>
<td>0.46 ± 0.14</td>
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</tbody>
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* Mean ± SE (n = 20 for each condition).

**Fig. 1. Release of nitrites.** Animals were given an i.t. dose of 0.9% NaCl solution or 3.6 mg/kg of BLM and sacrificed 24 h later. Cells were collected and purified as described in "Materials and Methods." Cocultures were established containing 3 x 10⁵ AM and 3 x 10⁵ L1210 cells and incubated for 18 to 24 h at 37°C in 10% CO2. Following incubation, the supernatant was assayed for the presence of nitrites as described in "Materials and Methods." In selected experiments, macrophages taken from control animals were activated in vitro for 24 h with LPS (5 μg) prior to the addition of L1210 cells and the 18-24 h coculture. A P < 0.05 was significant. *, P < 0.05. Columns, mean; bars, SEM (n = 23 for each condition).
vented the accumulation of nitrites in the coculture medium, RBC were added to the coculture and nitrites were measured. As with the NMA, our results show that, irrespective of the mode of activation, the presence of RBC in the coculture has a significant effect on the amount of measurable nitrites in the supernatant (Table 1). These results suggest that RBC may be modulating BLM-AM cytostatic activity by either inhibiting the production of nitrites or acting as a sink for the RNI produced by the AM.

Correlation of DNA Synthesis Inhibition in L1210 Cells with Nitrite Production by Alveolar Macrophages. We have reported that BLM-AM inhibit both thymidine incorporation and the activity of the mitochondrial enzyme aconitase in L1210 cells which could be completely abrogated by the RBC (24). To determine if the production of nitrites was related to the cytostatic activity of BLM-AM, we treated the BLM-AM:L1210 coculture with NMA. Fig. 2 confirms that only BLM-AM were cytostatic, and BLM-AM cytostatic activity was abrogated by both RBC and NMA. These results provide evidence supporting the hypothesis that the production of nitrites, likely RNI, is integrally related to the cytostatic activity of the BLM-AM.

To investigate if BLM-AM inhibition of aconitase activity was related to the production of nitrites, we measured the enzyme activity in L1210 cells cocultured with BLM-AM in the presence of NMA or RBC. Whereas the aconitase activity of L1210 cells cocultured with BLM-AM in the absence of RBC or NMA was significantly decreased as compared with L1210 cocultured with C-AM, the addition of either to the coculture system prevented BLM-AM-induced aconitase inhibition in the L1210 cell (Fig. 3).

**DISCUSSION**

Using an animal model for bleomycin-induced lung damage, we have demonstrated that RNI, measured as nitrites, are spontaneously released by BLM-AM. The production of the RNI appears to be critical for the action of BLM-AM against L1210 cells as measured by both decreased thymidine incorporation and aconitase activity. The presence of nitrites in the culture medium was diminished by both the substrate-specific inhibitor NMA and the RBC through an, as yet, undetermined mechanism.

Our finding that the RBC reduces the amount of measurable nitrites in the coculture has intriguing implications. There have been numerous reports in the area of vascular research which suggest that the molecule identified as EDRF is a form of NO and that the action of this molecule on smooth vessel walls is prevented by oxyhemoglobin and methemoglobin (21-23, 27, 28). Further, it has been shown that NO-mediated damage by AM is inhibitable by hemeproteins (20, 23). In a previous report we showed that the presence of RBC in the coculture abrogated the cytostatic effect of BLM-AM on target cells in a dose-dependent fashion (24). This finding suggests that, in whatever capacity the RBC are acting, there is a stoichiometric relationship between the protecting element(s) of the RBC and the effector molecule being generated by the macrophage. We also reported that the conversion of hemoglobin in the red cell to methemoglobin did not alter the effectiveness of this protection.

The similarities in the two systems are striking, and we hypothesize that, like oxyhemoglobin in the vascular model, hemoglobin in the intact RBC in our model is nitrosylated by the RNI and that the action of this molecule on smooth vessel walls is prevented by oxyhemoglobin and methemoglobin (21-23, 27, 28). Further, it has been shown that NO-mediated damage by AM is inhibitable by hemeproteins (20, 23). In a previous report we showed that the presence of RBC in the coculture abrogated the cytostatic effect of BLM-AM on target cells in a dose-dependent fashion (24). This finding suggests that, in whatever capacity the RBC are acting, there is a stoichiometric relationship between the protecting element(s) of the RBC and the effector molecule being generated by the macrophage. We also reported that the conversion of hemoglobin in the red cell to methemoglobin did not alter the effectiveness of this protection.

**Fig. 2. Correlation of DNA synthesis inhibition in L1210 cells with nitrite production by BLM-AM.** Animals were given an i.t. dose of 0.9% NaCl solution or 3.6 mg/kg of BLM and sacrificed 24 h later. Cells were collected and purified as described in "Materials and Methods." Cocultures were established as described in "Materials and Methods." In selected studies, 40 x 10⁶ RBC or 250 μM NMA, final concentration, was added to the cocultures. Results are expressed as the nmol of aconitase/min/10⁶ cells. Analysis of data is by analysis of variance. *P < 0.05. Columns, mean; bars, SEM (n = 12 for each condition).

**Fig. 3. Correlation of aconitase activity inhibition in L1210 cells and nitrite production by BLM-AM.** Animals were given an i.t. dose of 0.9% NaCl solution or 3.6 mg/kg of BLM and sacrificed 24 h later. Cells were collected and purified as described in "Materials and Methods." Cocultures were established as follows: 50 x 10⁶ AM were allowed to adhere for 2 h at 37°C in 10% CO₂, after which time the nonadherent cells were removed. L1210 cells, 5 x 10⁶, were added to each AM monolayer, and the cocultures were incubated for an additional 18 to 24 h. At the end of the incubation, the nonadherent cells, >85% L1210, were removed and assayed for aconitase activity as described in "Materials and Methods." In selected studies, 40 x 10⁶ RBC or 250 μM NMA, final concentration, was added to the cocultures. Results are expressed as the nmol of aconitase/min/10⁶ cells. Analysis of data is by analysis of variance. *P < 0.05. Columns, mean; bars, SEM (n = 12 for each condition).
action, adding further support to the notion that RNI are the active molecules in this model. We further demonstrated that both reduction of aconitase activity and DNA synthesis inhibition in the L1210 cell were related to nitrite production by BLM-AM. Both actions of BLM-AM were blocked by the addition of NMA to the coculture system.

Using LPS to activate C-AM in vitro, we addressed the relationship of nitrite production to cytostatic activity. Under the stimulatory effect of LPS, C-AM released nitrites into the culture medium. However, when incubated with RBC or NMA, although the production of nitrites is inhibited, DNA synthesis inhibition is not completely abrogated (data not shown). This suggests that the mechanism(s) activated by LPS are different from those activated by in vivo BLM-induced lung damage. Further investigation of in vivo models may therefore be relevant to understanding the role of the macrophage in inflammation and tumor management. In addition, the notion that BLM-induced lung damage may be mediated by RNI and that the production of RNI is inhibited by NMA and RBC suggests that the disease process may be amenable to biochemical and pharmacological manipulation.

As others have found, there is a relationship between the production of nitrites and the specific metabolic pattern of damage in target cells in this model (17, 19, 29, 30). This suggests that RNI are important in the cytostatic mechanism of macrophages activated in vivo by BLM-induced lung damage.

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REFERENCES

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