Macrophage Involvement in the Antitumor Activity of *Brucella abortus* Ether Extract against Experimental Lung Carcinoma Metastases

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ABSTRACT

*Brucella abortus* (strain 456) ether extract (Bru-Pel) and *B. abortus* lipopolysaccharide were tested for both in vitro and in vivo macrophage activation and antitumor activity in an artificial metastasis model. Purified cultures of resting macrophages were rendered nonspecifically tumoricidal for MBL-2 lymphoblastic leukemia cells by exposure to Bru-Pel at ≥1 ng/ml culture medium. *Brucella* lipopolysaccharide, in contrast to lipopolysaccharide extracted from *Escherichia coli* and *Salmonella typhimurium*, failed to activate macrophages in vitro at all concentrations tested (1 ng/ml to 10 μg/ml). However, Bru-Pel failed to activate macrophages from endotoxin-unresponsive C3H/HeJ mice as compared to potent activation of macrophages from closely related but endotoxin-responsive C3H/HeN mice. Treatment i.p. of homozygous nude mice with Bru-Pel induced cytotoxic macrophages, indicating that Bru-Pel activated macrophages through a thymus-independent process. An artificial metastasis model was developed in which single-cell suspensions derived from the M109 lung carcinoma were injected i.v. into BALB/c mice, and lung lesions were quantitated after 14 and 20 days by inflation of the lungs with India ink. Only Bru-Pel strikingly inhibited tumor nodule formation and was effective when given either 5 days prior to or 1 day after tumor inoculation. Histopathological studies of the lungs of Bru-Pel-treated mice revealed accumulations of macrophages surrounding the metastatic foci and arresting their development. Although Bru-Pel contains endotoxin, the data demonstrate that endotoxin is apparently not the active component by which Bru-Pel activates macrophages and enhances host resistance to neoplasia.

INTRODUCTION

Several bacteria or bacterial components have been observed to be potent stimulants of the macrophage system. Macrophages have been activated (rendered nonspecifically tumoricidal) in vivo by well-defined substances such as lipid A of gram-negative bacteria (2, 16) and peptidoglycan (or muropeptide) of bacterial cell walls (1, 13). Alexander and Evans (2) demonstrated that lipid A of endotoxin acted directly on macrophages without lymphocyte involvement. In addition, chronic infection with Mycobacterium tuberculosis or treatment with killed Corynebacterium parvum produced activated macrophages as a result of lymphokine secretion from thymus-derived lymphocytes (14, 24).

Bru-Pel has previously been shown to be a potent interferon inducer that enhances resistance to a variety of viral infections (6, 11). We have previously shown that polynucleonic interferon inducers and fibroblast-derived interferon preparations directly enhance macrophage tumoricidal function (19, 20). In this report we demonstrate that Bru-Pel activates macrophages and retards the development of artificially induced lung carcinoma metastases. Moreover, the active component of Bru-Pel appears to be separate from endotoxin.

MATERIALS AND METHODS

Mice

Male BALB/c × DBA/2 F, (hereafter called CD2F,), C3H/HeN, and BALB/c mice were obtained from the Mammalian Genetics and Animal Production Section of the NIH, Bethesda, Md. Male *nu/nu* mice were obtained from Charles River Breeding Laboratory, Wilmington, Mass. Male C3H/HeJ mice were purchased from Jackson Laboratory, Bar Harbor, Maine. Mice were housed in plastic cages and fed Purina laboratory chow and tap water *ad libitum*.

Drugs

Bru-Pel and *Brucella* LPS were generously provided by Dr. Julius Youngner, Department of Microbiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pa. The methods used for preparation of Bru-Pel and *Brucella* LPS have previously been described (6). *Escherichia coli* LPS (0127:B8) and *Salmonella typhimurium* LPS were purchased from Difco Laboratories, Inc., Detroit, Mich. Partially purified mouse L-cell interferon was obtained from Dr. Kurt Paucker, Department of Microbiology, Medical College of Pennsylvania, Philadelphia, Pa. Pyran copolymer (NSC 46015) was obtained from Dr. David Breslow of Hercules Research Center, Wilmington, Del. All biological and synthetic agents were made up in pyrogen-free Dulbecco's phosphate-buffered saline at pH 7.2.

Cell Cultures

Tumor Cells. Established cell lines of MBL-2 (H2*(C57BL/6)) murine leukemia and M109 (H2*(BALB/c)) murine alveolar carcinoma cells were maintained in RPMI-FCS. Viability of the cells was determined by the trypan blue exclusion test.

Peritoneal Macrophages. Noninduced peritoneal macrophages were obtained by peritoneal lavage of male CD2F, and C3H/HeJ mice. For the peritoneal lavage, 1 ml of 0.85% NaCl was gently instilled into the peritoneal cavity and 1 ml of 0.85% NaCl was collected after the first instillation. The collected fluid was further diluted 1:10 with RPMI-FCS and used as an inoculum in cell culture. The purity of the peritoneal cell preparation was confirmed by negative staining with toluidine blue O and the dye exclusion test.

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*The abbreviations used are:* Bru-Pel, *Brucella abortus* (strain 456) ether extract; LPS, lipopolysaccharide; RPMI-FCS, Roswell Park Memorial Institute Medium 1640 supplemented with 20% heat-inactivated (50° for 30 min) fetal calf serum, gentamicin (100 μg/ml), 0.075% NaHCO₃, and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer.

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Macrophages were harvested from treated or normal control mice as previously described (22) and washed in RPMI Medium 1640, and approximately $4 \times 10^5$ macrophages were seeded into 16-mm wells on tissue culture Cluster24 plates (Costar, Cambridge, Mass.) in 1.0 ml RPMI-FCS. The cultures were incubated for 90 min, and macrophage monolayers were washed thoroughly with jets of medium before use in experiments.

**Macrophage Activation Assay**

A modification of our previously described technique (19) was used to measure the ability of agents to induce macrophage-mediated cytotoxicity. Monolayers of macrophages in 16-mm wells were overlaid with $4 \times 10^4$ MBL-2 cells contained in 2.0 ml RPMI-FCS. For in vitro activation varying doses of drugs were immediately added to the cell mixtures and remained in the culture medium for the duration of the experiment. Toxicity controls consisting of MBL-2 cells alone in the presence of drug were also included in each experiment. All cultures were maintained in a humidified, 5% CO$_2$-in-air incubator at 37°, and cytotoxicity was assessed at 48 hr on the basis of viable cell counts in a hemocytometer. The ratio of macrophages to target cells was approximately 10:1 at the beginning of each experiment. The percentage of growth inhibition of MBL-2 cells due to macrophage-drug interaction was calculated by comparison to MBL-2 cells grown in the presence of resting macrophages alone.

**Inhibition of Artificially Induced Lung Metastases**

M109 cells grown in vitro were harvested during their exponential growth phase by gentle trypsinization, washed twice, and resuspended in serum-free RPMI Medium 1640. The number of single viable cells was determined and adjusted to $1 \times 10^6$ cells/ml medium. Tumor cells were injected i.v. into normal BALB/c mice. Inoculum volume per mouse was 0.2 ml (2 $\times 10^6$ cells). Drugs were given i.p. to randomized groups of mice either 5 days prior to, on the same day as, or 1 day following tumor cell inoculation. Controls received 0.2 ml Dulbecco's phosphate-buffered saline. Five mice from each group were sacrificed on Days 14 and 20, and the number of pulmonary metastases was determined by India ink inflation by the technique of Wexler (25).

**Histopathology**

Lungs were excised at different time intervals after tumor inoculation and fixed in 10% neutral formalin. These tissues were sectioned and routinely stained with hematoxylin and eosin.

**RESULTS**

**Direct Macrophage Activation in Vitro by Bacterial Agents.** Bru-Pel was compared to various LPS preparations for the ability to transform normal resting macrophages into cytotoxic effector cells in vitro (Chart 1). Bru-Pel activated macrophages from CD2F$_1$ mice to inhibit MBL-2 leukemia cell growth with optimal activity at 10 $\mu$g/ml, although significant activity remained at concentrations as low as 1 ng/ml. LPS preparations derived from *E. coli* and *S. typhimurium* showed kinetics similar to those of Bru-Pel. In contrast, LPS from *B. abortus* did not have any effect on resting macrophages at all concentrations tested. None of these preparations had a direct inhibitory effect on MBL-2 target cells in the absence of macrophages.

We also tested the effect of Bru-Pel, interferon, and *E. coli* LPS on macrophages from endotoxin-responsive C3H/HeN mice and closely related, but endotoxin-unresponsive, C3H/HeJ mice. Macrophages from both strains of mice were effectively stimulated by interferon at $1 \times 10^5$ units/ml culture medium (Chart 2). In sharp contrast, only C3H/HeN mice were activated by Bru-Pel and *E. coli* LPS at 10 $\mu$g/ml culture medium. However, the mechanism for this state of Bru-Pel unresponsiveness and the relationship of this defect to the endotoxin unresponsiveness of C3H/HeJ mice are not yet known.

**Macrophage Activation in Vivo by Bru-Pel.** We studied the effect of i.p. Bru-Pel treatment on macrophage reactivity from CD2F$_1$ mice. Pyran copolymer (25 mg/kg) served as a positive control (22, 23). Peritoneal macrophages potently inhibited MBL-2 cell growth when harvested 6 days after...
Bru-Pel treatment at 100 and 10 mg/kg (Table 1). A similar degree of activation was observed in macrophages from athymic nu/nu mice. No significant activation by Bru-Pel was obtained at 1 mg/kg.

**Effect of Bru-Pel on Experimental Pulmonary Metastases.** Pyran copolymer (NSC 46015) has previously been shown to retard the development of experimental M109 lung metastases (15, 17). For determination of whether Bru-Pel was similarly effective in potentiating surveillance against M109 pulmonary metastases, Bru-Pel was administered as a single i.p. injection either 5 days prior to, on the same day as, or 1 day after i.v. tumor inoculation. Mice were sacrificed on Days 14 and 20, and lungs were inflated with India ink to visualize tumor nodules. The results in Table 2 show that Bru-Pel almost completely suppressed the development of metastatic lung lesions. In contrast to Table 2 show that Bru-Pel almost completely suppressed the development of metastatic lung lesions. In contrast to multiple tumors in the lungs of placebo-treated animals, many lungs from Bru-Pel-treated mice remained tumor free at Day 20 (Fig. 1). Potent antitumor activity was demonstrated when Bru-Pel was given as a single injection during the period of 5 days before to 1 day after tumor inoculation (Table 2). Brucella LPS was without effect on experimental metastasis development.

**DISCUSSION**

The role of the macrophage in mediating nonspecific tumor resistance has gained considerable attention in recent years. Hibbs (9) advanced the theory that nonspecifically activated macrophages in the host provide an effective antitumor surveillance system by differentiating transformed cells from normal cells in a somatic population and by selectively killing these transformed cells by a secretion of lysosomal enzymes of macrophage origin into the cytoplasm of the susceptible cell (10). In addition, we have shown that the ability of adjuvants to enhance host resistance to cancer correlated with their capacity to produce cytotoxic macrophages (21, 23).

The significance of the activated macrophage to the study of metastasis has been suggested by several investigators. Mammalian solid tumors frequently contain a large number of resident macrophages that reach as high as 60% of the total cell population (5). The functional significance of these tumor-associated macrophages is yet to be clearly defined, although the percentage of these cells has been suggested to be directly proportional to the tumor's immunogenicity and inversely correlated with its metastatic potential (4). Fidler (7) presented evidence from an experimental metastasis model that the i.v. injection of specifically activated macrophages significantly reduced the number of established pulmonary metastases. Wood and Gillespie (26) showed that when fibrosarcoma-cell suspensions depleted of macrophages by adherence were injected s.c. into normal syngeneic mice, the tumors displayed an increased potential for metastasis. By contrast, control animals that received tumor-cell suspensions containing the normal complement of macrophages invariably developed progressive localized tumors. In addition, Bomford and Moreno (13) demonstrated a temporal correlation between the presence of cytostatic macrophages and lung metastasis inhibition after injection with C. parvum and a number of polysaccharides. These studies when taken collectively strongly suggest a regulatory role for the macrophage in tumor growth and metastasis.

Studies with adjuvants have further demonstrated the importance of macrophage concentration at the primary and disseminated tumor site. In studies with glucan and Bacillus Calmette-Guérin, it was found that intratumoral injection was the most efficacious route of therapy where macrophages accumulated at the site of inflammation, were activated locally, and killed tumor cells as innocent bystanders (8, 12). In contrast, i.p. pyran therapy causes an

### Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>% inhibition of MBL-2 cell growth&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyran</td>
<td>25</td>
<td>94 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bru-Pel</td>
<td>100</td>
<td>91 ± 1</td>
</tr>
<tr>
<td>Bru-Pel</td>
<td>10</td>
<td>98 ± 1</td>
</tr>
<tr>
<td>Bru-Pel</td>
<td>1</td>
<td>4 ± 6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Macrophages were harvested 6 days after i.p. drug treatment and were tested in vitro for ability to inhibit MBL-2 cell proliferation.  
<sup>b</sup> Mean ± S.E.

### Table 2

<table>
<thead>
<tr>
<th>Drug treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day 14</th>
<th>T/T&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Day 20</th>
<th>T/T&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.0 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5/5</td>
<td>117.0 ± 54.3</td>
<td>5/5</td>
</tr>
<tr>
<td>Pyran copolymer (Day -5)</td>
<td>0.6 ± 0.4</td>
<td>2/5</td>
<td>0.4 ± 0.2</td>
<td>2/5</td>
</tr>
<tr>
<td>Bru-Pel (Day -5)</td>
<td>0.0 ± 0.0</td>
<td>0/5</td>
<td>2.8 ± 0.9</td>
<td>4/5</td>
</tr>
<tr>
<td>Bru-Pel (Day 0)</td>
<td>0.6 ± 0.4</td>
<td>2/5</td>
<td>1.6 ± 0.8</td>
<td>3/5</td>
</tr>
<tr>
<td>Bru-Pel (Day 1)</td>
<td>0.4 ± 0.2</td>
<td>2/5</td>
<td>3.4 ± 2.7</td>
<td>3/5</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.4 ± 1.8</td>
<td>5/5</td>
<td>&gt;250</td>
<td>5/5</td>
</tr>
<tr>
<td>Pyran copolymer (Day -5)</td>
<td>1.2 ± 0.7</td>
<td>1/5</td>
<td>7.4 ± 4.6</td>
<td>2/5</td>
</tr>
<tr>
<td>Bru-Pel (Day -5)</td>
<td>0.2 ± 0.2</td>
<td>1/5</td>
<td>0.6 ± 0.4</td>
<td>2/5</td>
</tr>
<tr>
<td>Brucella LPS (Day -5)</td>
<td>5.0 ± 2.0</td>
<td>4/5</td>
<td>&gt;250</td>
<td>5/5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Drugs were administered i.p. at following doses: pyran (25 mg/kg); Bru-Pel (100 mg/kg); and Brucella LPS (2 mg/kg).  
<sup>b</sup> M109 cells (2 x 10<sup>5</sup>) were injected i.v. on Day 0. Lung lesions were identified by inflation of India ink.  
<sup>c</sup> T/T, mice with lung tumors/total number of mice.  
<sup>d</sup> Mean ± S.E.
influx of macrophages into the distal s.c. tumor site (21, 22). Moreover, i.p. pyran therapy produces macrophage accumulations and histiocytic granulomas in the lungs and protects mice from experimental metastasis formation (17).

Our finding of the effectiveness of Bru-Pel as a macrophage stimulator is in accordance with our previous reports on the involvement of interferon in enhancing macrophase tumoricidal function (19, 20). Endotoxin derived from B. abortus is a poor interferon inducer (6) and in this study did not significantly activate macrophages in vitro. In contrast, Bru-Pel has been shown to be a potent inducer of a "virus-type" interferon (6) and was quite active at producing cytotoxic macrophages. Although Alexander and Evans (2) showed that lipid A of gram-negative bacterial endotoxin activates macrophages, it appears that the lipid A of Enterobacteria is different from that of Brucella (18). This might explain the inability of B. abortus LPS to activate macrophages and enhance surveillance against metastatic foci in the lungs.

Pretreatment of mice with Bru-Pel but not Brucella LPS potently inhibited lung metastasis formation. Preliminary histopathological observations of the host response to tumor give further support to the role of the activated macrophage in tumor surveillance. Macrophage accumulations in the interstitium of the lungs from Bru-Pel-treated animals were observed to encircle the metastatic foci and completely arrest their development. Our current data support the concept that activated macrophages have a surveillance function in inhibiting or controlling metastatic cell growth. Moreover, the enhancement of macrophage numbers and activity in the lungs following systemic Bru-Pel treatment could provide an approach to possible treatment.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Effect of Bru-Pel and B. abortus LPS treatment on artificially induced M109 pulmonary metastases. Mice were treated i.p. with Bru-Pel (100 mg/kg) or Brucella LPS (2 mg/kg) 5 days prior to receiving 2 × 10⁶ M109 cells i.v. Some mice received 0.2 ml Dulbecco's phosphate-buffered saline as placebo. Top row, India ink-inflated lungs from placebo-treated animals on Day 20. Numerous tumor lesions are visualized by India ink treatment. In contrast, the middle row shows lungs that are essentially free of identifiable tumor nodules from Bru-Pel-treated animals. Lungs from Brucella LPS-treated mice (bottom row) appeared like placebo-treated controls.
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