Activation of Rat Alveolar Macrophages to the Tumoricidal State in the Presence of Progressively Growing Pulmonary Metastases

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ABSTRACT

The purpose of this study was to determine whether the presence of progressively growing pulmonary metastases influences the number and function of alveolar macrophages (AM). Female F344 rats were given i.v. injections of cells from a metastatic variant line of the syngeneic adenocarcinoma MADB-105. At Days 7, 14, 21, and 28 after injection, normal and tumor-bearing animals (3/group) were killed, and their AM were harvested by lavage. The functional integrity of AM was determined by their capacity to phagocytose opsonized erythrocytes and by their ability to respond to a variety of activating agents in vitro. Normal and metastasis-bearing rats were given i.v. injections of Nocardioid rubra cell wall skeleton to determine whether the presence of large pulmonary metastases would interfere with AM activation in situ. The data demonstrated that the presence of progressively growing lung metastases led to a slight increase in the number of harvested AM and that these cells from tumor-bearing rats were as phagocytic as AM obtained from normal rats. Also, AM harvested from rats with metastases became tumoricidal against syngeneic tumor target cells in response to activation stimuli in vitro and in vivo.

We conclude that, in this tumor system, the presence of progressively growing lung metastases does not suppress the number or function of AM. The use of agents known to activate AM could, therefore, be of benefit in the therapy of pulmonary metastases.

INTRODUCTION

AM* function as primary effector cells against microorganisms that reach the lungs. However, their exact function in the control of primary and/or metastatic pulmonary tumors is less clear. The lung is the most frequent site for the development of metastases, and the growth of metastatic tumor cells in the lung is influenced by both tumor cell properties and host factors (11, 26). Experimental studies of metastasis demonstrated that, although most circulating tumor cells reach the lung vasculature (9, 20), the vast majority of arrested tumors are destroyed and only a few cells survive to proliferate into clinically detectable lesions. Little is known about the mechanism of this phenomenon.

The functional integrity of AM may be important in the destruction of tumor cells reaching the lungs. AM can be rendered tumoricidal following their interaction with bacteria such as Corynebacterium parvum (19, 25) or Bacillus Calmette-Guerin (38) and bacterial products such as LPS (32), N-CWS (34), or MDP (33). Moreover, AM lavaged from lungs of normal rats can respond to free and/or liposome-encapsulated lymphokines to become tumoricidal (35). The tumor-cytotoxic properties of these activated AM are similar to those of tumoricidal peritoneal macrophages (1, 6, 7, 13, 17).

In several experimental systems, the presence of progressively growing tumors has been shown to be accompanied by several alterations in macrophage function, such as enhancement of carbon clearance in vivo (4, 24), increased expression of monocyte Fc receptors (28, 29), depression of delayed cutaneous hypersensitivity (3, 8), and suppression of migration or chemotactic response of macrophages from the peritoneal cavity or the site of tumor growth (3, 8, 21–23, 28–31). Pulmonary tumor growth has been shown to affect certain AM properties (15), although the effect of tumor burden on other characteristics of these host cells is still unknown. Namely, does the presence of growing metastases suppress the migration of blood monocytes into the lung parenchyma? Can AM in hosts bearing pulmonary metastases respond to exogenous activation stimuli and become tumoricidal? The answers to these questions may have clinical implications since the absence of AM or their failure to respond to activation stimuli in metastasis-bearing hosts would limit the usefulness or efficacy of immunotherapeutic agents for the control of pulmonary metastases.

In the present report, we demonstrate that, in the lungs of rats bearing progressively growing metastases from a syngeneic mammary adenocarcinoma, the number of AM is increased. Moreover, AM obtained from metastasis-bearing rats responded to activation stimuli and were as phagocytic and tumoricidal as AM from normal control donors.

MATERIALS AND METHODS

Animals. Specific pathogen-free inbred female F344 rats, 5 to 7 weeks old, were obtained from the Frederick Cancer Research Center's Animal Production Area.

Cell Lines. Syngeneic tumors, MADB-100 and MADB-200, are mammary adenocarcinomas induced in different F344 rats by a single dose (20 mg) p.o. of 9,10-dimethyl-1,2-benzanthracene (Sigma Chemical Co., St. Louis, Mo.). MADB-105 is a selected variant cell line obtained from a pulmonary metastasis produced by the i.v. injection of MADB-100 tumor cells. In vitro assays were always done with cells from cultures in their exponential growth phase. All monolayer cultures were main-
tained in Eagle's minimal essential medium supplemented with 5% fetal bovine serum, vitamins, sodium pyruvate, nonessential amino acids, and L-glutamine (Flow Laboratories, Rockville, Md.) at 37°C in a humidified atmosphere of 5% CO₂. The media did not contain endotoxins, as determined by the Limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, Mass.). All cultures were free of Mycoplasma, reovirus type 3, pneumonia virus of mice, K-virus, Theller's encephalitis virus, Sendai virus, minute virus of mice, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ecromelia virus, and lactate dehydrogenase virus (MA Bioproducts, Walkersville, Md.).

Reagents. LPS (Escherichia coli 055:B5) was purchased from Difco Laboratories, Detroit, Mich. N-CWS was prepared by methods described previously (37); 1 mg of N-CWS or N-CWS placebo preparations consisted of squalene (2 mg), mannitol (14.1 mg), and Tween 80 (0.5 mg). The N-CWS preparations (Fugisawa Pharmaceutical Company, Osaka, Japan) were a gift of Professor Yuichi Yamamura (Osaka University, Osaka, Japan). MDP was purchased from Calbiochem, La Jolla, Calif. The MDP did not contain endotoxins, as detected by the Limulus amebocyte lysate assay.

MAF. Endotoxin-free (Limulus amebocyte lysate assay) and cell-free supernatants rich in MAF activity were harvested from cultures of rat lymphocytes stimulated in vitro with concanavalin A bound to Sepharose beads (Pharmacia Fine Chemicals, Piscataway, N. J.), as described previously (13). The supernatants were filtered through a 0.22-mmol Millipore membrane and stored at -70°C until use. For convenience and brevity, cell-free culture supernatants that are rich in MAF activity will be referred to simply as MAF throughout the remainder of this paper.

Lipids and Preparation of Liposomes. Chromatographically pure egg phosphatidylcholine and bovine brain phosphatidylserine were purchased from Avanti Biochemicals, Birmingham, Ala. Multilamellar vesicles (liposomes) were prepared from a mixture of phosphatidylcholine and phosphatidylserine (7:3 M ratio) by mechanical vortex shaking. Encapsulation of MDP or HBSS within liposomes was achieved by using methods similar to those described previously (27). Liposome preparations were adjusted to 1 μmol total lipid per ml in supplemented media and used within 24 hr of preparation. The experiments reported here have been done with multilamellar vesicles containing encapsulated aqueous material added to macrophages at a liposome concentration of 100 nmol phospholipids per 10⁵ cells. This dose of liposomes contains approximately 0.24 μl of encapsulated material. The dose of liposomes used routinely to render AM tumoricidal was not toxic to target cells (27).

Induction of Experimental Metastasis. Six-week-old female F344 rats were given i.v. injections of 2 x 10⁵ viable MADB-105 cells. At 7, 14, 21, and 28 days after injections, AM were harvested by lavage. In all experiments, AM donor groups consisted of 3 to 5 rats. Following the lavage for AM, lungs were fixed in Bouin's solution to facilitate visualization of metastases (11).

In Vivo Activation of Rat AM by N-CWS. Normal and metastasis-bearing rats were given i.v. injections of 300 μg of N-CWS preparation or placebo. Twenty-four hr later, the rats (3 per group) were anesthetized, and their AM were harvested as described below. The tumoricidal properties of these AM were assayed by the addition of radiolabeled target cells 60 min after AM plating in vitro (see below).

Harvest and Purification of AM Cultures. AM were obtained by a tracheobronchial lavage method described fully elsewhere (35). Briefly, F344 rats were anesthetized with an i.p. injection of pentobarbital sodium and exsanguinated by severing both renal arteries. After opening the chest cavity to produce pneumothorax, the trachea was cannulated with a cut tube from a Butterfly-19 or -21 infusion set (Abbott Laboratories, Chicago, III.) and anchored by suturing. The lungs were lavaged with 5 ml of Ca²⁺-Mg²⁺-free HBSS prewarmed to 37°C. The process was repeated several times to yield a recovered total of 50 ml lavage fluid per rat. The total number of cells collected was determined by counting with a hemocytometer (using 2% acetic diluent), and the viability of nucleated cells (in HBSS suspension) as assessed by trypan blue dye exclusion was >95%. The lavaged cells from normal rats consisted of >97% AM (nonspecific esterase positive). The remaining cells were small mononuclear cells or neutrophils, which were eliminated during washing of plated cells (see below). The lavage suspension was washed and resuspended in serum-free media, and 10⁶ AM were plated into wells of a Microtest II plate with a surface area of 38 sq mm (Falcon Plastics, Oxnard, Calif.). Nonadherent cells (less than 10%) were removed by a media wash 60 min after the initial plating. For in vitro activation, the AM were incubated for 24 hr with either media alone or media containing activating agents (LPS, MAF, N-CWS). This step was eliminated when macrophages obtained after activation in vivo were used as effector cells in the cytotoxicity assays. Twenty-four hr later, the cultures were washed with medium. At that time, >99% of the adherent cells exhibited mononuclear morphology and phagocytosed carbon particles.

Assay of AM-mediated Cytotoxicity in Vitro. AM-mediated cytotoxicity was assessed by a radioactive release assay detailed previously (35). Target cells in exponential growth phase were incubated for 24 hr in complete minimal essential medium supplemented with 5×10⁴[¹²⁵I]jodo-2'-deoxyuridine (0.2 μCi/ml) (specific activity, 200 μCi/mmol; New England Nuclear, Boston, Mass.). The target cells were then washed with warm HBSS to remove unincorporated radiolabel, harvested by trypsinization (0.25% trypsin:0.02% EDTA for 1 min at 37°C), and resuspended in media, and 1 x 10⁴ target cells were plated per well to obtain an initial AM:target cell ratio of 10:1. At this population density, normal (untreated) AM are not cytotoxic to neoplastic cells, whereas activated AM are. No significant differences were detected in the plating efficiency of 125I-labeled target cells to the plastic or to monolayers of AM (normal or activated). Radiolabeled target cells also were plated alone as an additional control group. All cultures were refed 4 hr after the plating of tumor cells. The AM target cell cultures were then incubated in complete minimal essential medium at 37°C. One- or 3-day cultures were washed twice with HBSS to remove dead and dying nonadherent cells, and the adherent (viable) cells were lysed with 0.1 ml of 0.5 N NaOH. The lysate was absorbed in a cotton swab, and radioactivity was measured in a gamma counter. Maximal in vitro macrophage-mediated cytotoxicity in this assay is obtained after 3 days of incubation with target cells, and macrophages do not reincorporate 5×10⁴[¹²⁵I]jodo-2'-deoxyuridine released from dead target cells (10). The cytotoxic activity of the macrophages was calculated as
follows.

% of cytotoxicity

\[
\frac{(cpm \text{ in target cells cultured with normal macrophages}) - (cpm \text{ in target cells cultured with test macrophages})}{\text{cpm in target cells cultured with normal macrophages}} \times 100
\]

Quantitative Assay of Phagocytosis. Opsonized sheep RBC labeled with \(^{51}\text{Cr}\) (0.2 ml of 0.4% suspension) were added to \(10^6\) AM plated into culture wells (14). Following a 2-hr incubation at 37\(^\circ\), the cultures were rinsed once for 10 sec with distilled water to lyse nonphagocytosed RBC and washed twice with HBSS. The remaining adherent cells were lysed with 0.5 n NaOH, and the lysate was monitored for radioactivity in a gamma counter (14). Values were obtained from data on triplicate cultures.

Statistical Analysis. Experimental results were analyzed for their statistical significance by Student’s \(t\) test (2-tailed).

RESULTS

The i.v. injection of \(2 \times 10^5\) viable MADB-105 adenocarcinoma cells produced a large number of pulmonary metastases. By Day 7, the median number of grossly obvious metastases was 35 (range, 0 to 89); on Day 14, it was 58 (range, 9 to 136); on Day 21, it was 67 (range, 21 to 219); and on Day 28, the median number of pulmonary metastases was 103 (range, 39 to 306). In Fig. 1, we show the histological appearance of lungs collected from rats at various times after i.v. injection of MADB-105 cells. Lung colonies were detectable under the dissecting microscope by 1 week postinjection. With time, most metastases (colonies) increased in size, and, by 4 to 5 weeks postinjection, tumor tissue had obliterated or occupied a large proportion of the lung parenchyma.

Effect of Progressing Metastases on the Number of Recovered AM. At 7, 14, 21, and 28 days after i.v. injection of tumor cells, normal and tumor-bearing rats (3 per group) were killed, their lungs were lavaged, and the number of recovered AM was determined by microscopy. Data from 3 independent experiments are shown in Chart 1. The average yield of AM from 8- to 10-week-old normal F344 rats was approximately \(2 \times 10^4\) AM per g body weight. The presence of progressively growing metastases did not diminish the number of AM. In fact, the number of AM recovered from the lungs of rats bearing metastases was slightly higher than that recovered from age-matched controls. In normal rats, more than 97% of the lavaged AM were AM, as determined by nonspecific esterase staining. In metastasis-bearing rats, 94 to 96% of the lavaged cells were AM. The presence of tumor cells in the lavaged cell suspension was not detected until 4 to 5 weeks following the i.v. injection of cells, i.e., 1 week longer than the longest time studied.

Phagocytic Capacity of AM Harvested from Metastasis-bearing Rats. AM were harvested from groups of rats (\(n = 3\)) at various times after the i.v. injection of \(2 \times 10^5\) MADB-105 cells. The combined data from 3 independent experiments are summarized in Table 1 and show no differences in phagocytic capacity between AM harvested from normal rats and those from metastasis-bearing rats.

The Response of AM to in Vitro Activation and Acquisition of Tumoricidal Properties. AM lavaged from lungs of normal rats or those bearing pulmonary metastases were incubated in vitro for 24 hr in media alone (control) or media containing different macrophage activators that included LPS (5 \(\mu\)g/ml), MDP (50 \(\mu\)g/ml), N-CWS (1 \(\mu\)g/ml), MAF-free preparation (unencapsulated), and liposome-encapsulated MAF. Following the activation period, the AM preparations were washed and assayed for development of tumoricidal capacity. Without any in vitro activation (AM incubated in media), neither AM from normal nor AM from tumor-bearing rats were cytotoxic to either MADB-200 or MADB-105 target cells (Table 2). However, AM from normal rats and AM from rats bearing metastases for 7, 14, 21, or 28 days responded alike to activation by LPS, MDP, N-CWS, free MAF, and/or liposome-encapsulated MAF (Table 2). These AM were highly cytotoxic to both syngeneic tumor target cells in vitro.

In Vivo Activation of Tumoricidal Properties in AM from Metastasis-bearing Rats. The above data demonstrated that, once removed from the lungs, AM from tumor-bearing rats responded to activation stimuli in vitro. A more meaningful result, however, would be the demonstration that AM present in the lungs of rats with large metastases can be activated to become tumoricidal in situ. To study this possibility, rats bearing metastases (as well as normal rats) were given i.v. injections of 300 \(\mu\)g/rat of either N-CWS or placebo preparations. Twenty-four hr following this treatment, AM were harvested and purified (see “Materials and Methods”), and their tumoricidal properties were assayed in vitro. AM harvested from untreated normal or metastasis-bearing rats were not tumoricidal in vitro against MADB-105 cells (Table 3) or MADB-200 cells (data not shown). The i.v. injection of placebo preparations did not activate AM. In contrast, the i.v. injection of 300 \(\mu\)g of N-CWS preparation rendered AM highly cytotoxic against tumor target cells (\(p < 0.001\)). AM from both normal and tumor-bearing rats were equally cytotoxic, suggesting that, even in rats bearing large metastases (see Fig. 1, 28 days after
DISCUSSION

Our present studies demonstrate that the number of recoverable rat AM and their ability to respond to activation stimuli such as LPS, MDP, N-CWS, MAF in vitro and N-CWS in vivo to become tumorcidal are not diminished in the presence of progressively growing pulmonary metastases of a syngeneic mammary adenocarcinoma.

The establishment and growth of metastases are determined by the outcome of 2 conflicting factors: tumor cell multiplication and tumor cell destruction. When the rate of tumor cell division exceeds the rate of tumor cell destruction, then visible metastases develop (11). The extent of tumor cell destruction by host immune cells, including macrophages, is regulated by at least 2 factors: the ratio of effector cell to target cells and the efficiency with which individual effector cells destroy targets. Thus, if macrophages are important in the control of pulmonary metastases (9, 12, 33–35), the progressive growth of these lesions may be accompanied by a decrease in the number of AM, a decrease in their functional ability, or both.

The migration of macrophages into the peritoneal cavity of mice, induced by the i.p. injection of various stimulants, is reduced in animals bearing tumors at other sites (3, 21–23, 28–31). Such was not the case in our studies. The number of AM recovered by lavage of the lungs increased in rats bearing metastases as compared with age-matched normal controls. These data are in agreement with a previous study using allogeneic tumors (15). Because AM are probably derived from bone marrow macrophage precursors and of peripheral blood monocytes is sometimes increased in tumor-bearing animals (2, 23), an increase in the number of AM in lungs bearing metastases is not unexpected. The phagocytic capabilities of AM recovered from lungs of rats with small or large metastases were similar to those harvested from normal rats. These data agree with some (21, 22), but not all (15), previous reports dealing with the function of macrophages obtained from tumor-bearing animals.

Macrophages can be rendered tumorcidal by a variety of agents, including lymphokines, such as MAF released by antigen- and/or mitogen-sensitized lymphocytes (6, 10, 13, 18). Several recent studies (10, 18) have suggested that, in mice bearing a progressively growing tumor, the interaction between lymphocytes and macrophages fails to occur. Thus, lymphocytes from mice bearing large progressive tumors failed to produce MAF when challenged with tumor cells in vitro but were still capable of releasing MAF in response to unrelated

### Table 2

<table>
<thead>
<tr>
<th>Time (days) after i.v. injection of tumor cells</th>
<th>% of AM-mediated cytotoxicity (%)</th>
<th>Media</th>
<th>LPS (5 µg/ml)</th>
<th>MAF (free)</th>
<th>Liposome-encapsulated MAF</th>
<th>MDP (50 µg/ml)</th>
<th>N-CWS (1 µg/ml)</th>
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<tr>
<td></td>
<td>Donor rats</td>
<td></td>
<td></td>
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<td>7</td>
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<td>60 c</td>
<td>55 e</td>
<td>47 f</td>
<td>32 g</td>
<td>85 i</td>
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<td></td>
<td>Tumor bearing</td>
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<td>35</td>
<td>47</td>
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<td>68</td>
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<td>52</td>
<td>29</td>
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<td>35</td>
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<td>Tumor bearing</td>
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<td>71</td>
<td>34</td>
<td>52</td>
<td>49</td>
<td>76</td>
</tr>
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</table>

a Rats were given i.v. injections of 2 x 10^5 viable MADB-105 cells, with 3 rats/group.

### Table 3

<table>
<thead>
<tr>
<th>Time (days) after i.v. injection of MADB-105 cells</th>
<th>% of AM-mediated cytotoxicity (%)</th>
<th>Donor rats</th>
<th>Placebo (300 µg/rat)</th>
<th>N-CWS (300 µg/rat)</th>
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<td>0 a</td>
<td>0 c</td>
<td>80 d</td>
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<tr>
<td></td>
<td>Tumor bearing</td>
<td>0</td>
<td>0</td>
<td>44</td>
</tr>
</tbody>
</table>

a Rats were given i.v. injections of 2 x 10^5 viable MADB-105 cells, with 3 rats/group.

b AM (1 x 10^5) were plated into Microtest II wells. The AM were incubated for 24 hr with media (untreated), LPS (5 µg/ml), MDP (50 µg/ml), N-CWS (1 µg/ml), free MAF (200 µg), or liposome-encapsulated MAF (100 nmol of multilamellar vesicles). The AM were thoroughly washed before the addition of 5-[125]iodo-2'-deoxyuridine-labeled MADB-105 target cells. Mean of triplicate cultures terminated 72 hr after the addition of 5-[125]iodo-2'-deoxyuridine-labeled MADB-105 cells. Similar results were obtained with MADB-200 cells (data not shown).

c Percentage of cytotoxicity as compared with tumor cells alone.

d Percentage of cytotoxicity as compared with control AM and tumor cells (p < 0.01).

i.v. injection), AM can respond in situ to activation by N-CWS (Table 3).
antigen or mitogen (10, 18). The inability of these lymphocytes to produce mediators that will activate macrophages in vitro suggests that an important amplification mechanism may be absent in these animals, as they may be unable to activate other host cells to destroy tumor cells. Previous studies from our laboratory (18) demonstrated that the lack of cytotoxicity of peritoneal macrophages harvested from mice bearing large syngeneic s.c. tumors was not due to an innate deficiency of the macrophages but rather to the lack of production of MAF by autologous lymphocytes. Similarly, macrophages isolated from progressing Moloney virus-induced sarcomas in BALB/c mice were found to be noncytotoxic in vitro, whereas those obtained from regressing tumors were cytotoxic in vitro (30). In this experimental system, noncytotoxic macrophages could be rendered tumoricidal following incubation with ng/ml quantities of LPS (30). These data suggest that macrophages in tumor-bearing animals could be activated in vivo in response to an exogenous activation stimulus. The data shown here, that the i.v. injection of N-CWS leads to the generation of the tumoricidal state in AM, support this hypothesis.

The question of whether AM present in lungs with progressively growing metastases can respond to activation stimuli is of obvious importance. Failure of AM to respond would restrict their role in cell-mediated immunological reactions in the lungs. Our present findings indicate that AM in rats bearing small or large metastases can respond to activation stimuli in vitro (LPS, MDP, MAF) and in situ (N-CWS). Following treatment with such agents, the AM are rendered tumoricidal and destroy in vitro syngeneic tumor cells. These findings confirm earlier studies in mice bearing tumors growing s.c. In such mice, peritoneal (10, 18, 21) or intratumoral macrophages (16, 30, 36) were found to be noncytotoxic to the tumor targets when examined by in vitro assays. In all 3 studies, however, such noncytotoxic macrophages could be rendered tumoricidal following in vitro incubation with either lymphokines (10, 18) or endotoxins (30).

In summary, our studies show that the presence of progressively growing lung metastases in this tumor system does not suppress the number or function of AM. Therefore, the systemic administration of agents that can activate AM in situ could be of benefit in the therapy of pulmonary metastasis.

REFERENCES

Fig. 1. The formation of pulmonary metastases. MADB-105 cells were injected i.v. into syngeneic rats. The rats receiving injections were killed at different timepoints thereafter, and their lungs were fixed and serially sectioned. A, 7 days postinjection; B, 14 days postinjection; C, 21 days postinjection; and D, 28 days postinjection. × 3.7.
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