Inhibition of Pulmonary Metastasis by Intravenous Injection of Specifically Activated Macrophages

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SUMMARY

The ability of syngeneic macrophages from C57BL/6 mice bearing a progressively growing B16 melanoma to inhibit established pulmonary metastases in vivo was studied. Macrophages were cultured in vitro with supernatants obtained from cultures of B16 tumor cells alone or tumor cells and either normal, nonspecifically sensitized or specifically sensitized xenogeneic (rat) lymphocytes. The different groups of in vitro-treated macrophages were injected i.v. or i.p. into other C57BL/6 mice that had been given i.v. injections of 10,000 viable B16 melanoma cells 48 hr previously.

The data demonstrated that specifically in vitro-treated macrophages injected i.v. but not i.p. into mice significantly reduced their number of established pulmonary metastases. Moreover, it appeared that the in vivo inhibition of tumor nodules was continuing at the time of sacrifice.

These results support the experimental data that cytotoxic macrophages may occupy an important role in the defense against neoplasia. Furthermore, the application of xenogeneic activation of macrophages from tumor-bearing animals rendering them cytotoxic may provide a possible approach to therapy.

INTRODUCTION

The macrophage plays an important role in the specific immunity to neoplasia (for review, see Ref. 1). In vitro cytotoxicity mediated by macrophages had been demonstrated in a variety of tumor systems following cell-to-cell interaction (1, 4). Mouse macrophages activated in vivo by infection of donors with Toxoplasma gondii were reported to be selectively cytotoxic to tumor cells by non-immunological mechanisms, i.e., such activated macrophages were cytotoxic in vitro only to transformed allogeneic mouse fibroblasts while not affecting normal allogeneic cells. These results suggested that the selective in vitro toxicity mediated by the activated macrophages was directed against abnormal growth characteristics of cells rather than their antigenic composition (11, 12). Macrophages can be nonspecifically activated to have enhanced antibacterial properties (14). The mechanisms involved in such activation have been reported to be specific, i.e., sensitized lymphocytes, upon reaction with an antigen, release products that nonspecifically activate the macrophage (2, 10, 14, 15).

Normal macrophages, while not demonstrably cytotoxic to tumor cells, can be "armed" (made specifically cytotoxic) by supernatants derived from in vitro cultures of mixed syngeneic spleen cells sensitized in vivo and tumor cells, or by incubating the normal macrophages with sensitized syngeneic thymocytes (1, 5, 10, 13). Once activated, the macrophage demonstrates increased adherence to glass, as well as increased mobility, phagocytic capability, and enzymatic activity (15). Such "armed" macrophages are also cytotoxic in vitro to tumor cells (1, 4, 5, 10, 13).

We have recently confirmed the hypotheses by Prehn (16, 17) that the normal immune response to neoplasia might have a dual role. During the early development of cancer or with weakly immunogenic tumors, the cell-mediated response might directly stimulate rather than inhibit tumor growth. Indeed, several studies of immune stimulation to tumor growth in vitro (8) and in vivo (16) have been reported. In addition, we have recently demonstrated that a low number of normal or sensitized syngeneic lymphocytes mixed in vitro with the B16 melanoma could increase the incidence of pulmonary metastasis in C57BL/6 mice given i.v. injections of the mixture. However, once a critical dose of immune cells is exceeded, cytotoxicity or inhibition of tumor metastasis was demonstrated (9). The growth and spread of cancer from a primary site to distant organs is a complicated biological phenomenon; as we demonstrated recently, tumor cells that readily metastasize might do so because they possess characteristics allowing for their survival (7). Immune mediated (lymphocytic) stimulation to metastasis may be but 1 method by which cancer cells are aided in their dissemination in vivo. Lymphocytes and macrophages are known to cooperate in the mediation of cellular cytotoxicity (1, 4, 5, 14). However, the role of the normal or activated macrophage in the phenomenon of immune stimulation to tumor growth is...
unknown. Specifically, do macrophages cooperate in mediation of cytotoxicity only, or do they aid lymphocytes in stimulation of tumor growth? Moreover, can normal or activated syngeneic macrophages abrogate the stimulation to tumor growth mediated by low numbers of syngeneic lymphocytes?

This report is concerned with several studies of the inhibitory effects of syngeneic mouse macrophages injected i.v. into mice bearing established metastases. The macrophages were activated in vitro by supernatants obtained from cultures of nonsensitized control, specifically or nonspecifically sensitized xenogeneic rat lymphocytes, and the B16 melanoma. The studies describe the results obtained in the early phases of our study and demonstrate the role of the specifically activated syngeneic macrophages in the inhibition of established tumor metastasis in vivo.

MATERIALS AND METHODS

Animals. Inbred mouse strains C57BL/6 (H-2b) and A (H-2a) were obtained from The Jackson Laboratories, Bar Harbor, Maine. Fischer 344 rats were supplied by Microbiological Associates, Inc., Bethesda, Md.

Tumors. The transplantable B16 melanoma, originating in C57BL/6 mice, and a.c. 15091, originating in A mice, were obtained from The Jackson Laboratories.

Sensitization in Vivo of Fischer Rats to Mouse Transplantation and Tumor Antigens. Fischer rats (150 g) were given 2 s.c. injections, 1 week apart, of 5 x 10⁸ viable tumor cells (B16 melanoma or a.c. 15091) or 2 x 10⁸ C57BL/6 mouse glass-wool-filtered spleen and lymph node cells. One week after the 2nd injection, the rats were killed, and their spleens and lymph nodes were collected aseptically.

B16 Melanoma Culture. The transplantable B16 melanoma was originally adopted to growth in tissue culture as described previously (6-8). Stock cultures were maintained in CMEM (Grand Island Biological Corp., Grand Island, N. Y.). The cells were cultured in a humidified 37° incubator containing 5% CO₂ atmosphere.

Macrophage Culture. Macrophages were obtained from C57BL/6 mice bearing the B16 melanoma growing progressively s.c. Two ml of thioglycollate were injected i.p. into each mouse, and 4 to 5 days later the mice were killed and their peritoneal exudate cells were harvested by washing with HBSS containing heparin, 2 units/ml. The cells were centrifuged and resuspended in CMEM; 20 x 10⁶ C57BL/6 cells/flask, 20 ml CMEM). After 24 hr, the supernatants of these cultures as well as those from B16 melanoma alone were collected, centrifuged at 600 rpm for 10 min and filtered through a 0.2-µm Millipore filter. The cell-free supernatants were then added to the 3- to 4-day-old monolayers of C57BL/6 macrophages that had been obtained from mice bearing the B16 melanoma s.c. After 48 hr, the macrophages were washed with CMEM and harvested with the aid of a soft, wide-tipped rubber policeman. Cellular counts and viability were determined, and the macrophages were placed into cold HBSS to prevent clumping.

In Vivo Induction of Macrophage Activation. Splens and lymph nodes from normal Fischer rats or those sensitized in vivo against C57BL/6 lymphocytes, B16 melanoma, or a.c. 15091 were collected aseptically, placed into HBSS, and pressed through a wire mesh sieve. The resulting suspensions were filtered through a 10-ml glass wool column and centrifuged, and the cellular pellets were resuspended in CMEM. Cellular viability as determined by the trypsin blue exclusion test was about 95%, and most cells appeared to be lymphocytes. The various rat non-glass-adherent lymphocytes were added to B16 melanoma nonconfluent monolayers at the ratio of 100:1 (2 x 10⁶ B16 cells/flask, 20 ml CMEM). After 24 hr, the supernatants of these cultures as well as those from B16 melanoma alone were collected, centrifuged at 600 rpm for 10 min and filtered through a 0.2-µm Millipore filter. The cell-free supernatants were then added to the 3- to 4-day-old monolayers of C57BL/6 macrophages that had been obtained from mice bearing the B16 melanoma s.c. After 48 hr, the macrophages were washed with CMEM and harvested with the aid of a soft, wide-tipped rubber policeman. Cellular counts and viability were determined, and the macrophages were placed into cold HBSS to prevent clumping.

Procedures for the Study of Experimental Metastasis and the In Vivo Effects of Activated Macrophages. B16 melanoma cells grown in vitro were harvested during their exponential growth phase by a short trypsinization (0.25% trypsin: EDTA solution for 1 min), washed twice, and resuspended in HBSS. The number of single viable tumor cells was determined and adjusted to 50,000 cells/ml HBSS. Tumor cells were injected i.v. into the tail vein of normal C57BL/6 mice. Inoculum volume per mouse for all experiments was 0.2 ml (10,000 cells); all mice were placed back into 1 large cage. After 48 hr, the mice were randomly divided into several treatment groups and injected i.v. with either 0.9% NaCl solution or macrophages from 1 of the several treated groups (Tables 1 and 2). The groups were coded, and 2 or 3 weeks later all mice were killed. The number of subsequent pulmonary metastases was determined with the aid of a dissecting microscope by 2 independent observers.

Statistical Analysis. This was carried out by Student's t test.
RESULTS

Macrophages were harvested from C57BL/6 mice bearing a progressively growing B16 melanoma s.c. These macrophages were cultured in vitro with various supernatants obtained from xenogeneic lymphocytes after their interaction with the tumor in vitro or with supernatants from tumor cultures alone. Following this incubation the macrophages were injected i.p. or i.v. into C57BL/6 mice that 48 hr previously had been injected i.v. with 10,000 viable B16 cells. The in vitro effects of these syngeneic macrophages are summarized in Tables 1 and 2.

Table 1

<table>
<thead>
<tr>
<th>Macrophage treatment in vitro</th>
<th>i.v.*</th>
<th>i.p.†</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Tumor cells alone</td>
<td>205 ± 45 (150–291)</td>
<td>205 ± 45 (150–291)</td>
</tr>
<tr>
<td>B. Macrophages incubated with supernatants of B16 melanoma cultures</td>
<td>227 ± 36 (185–261)</td>
<td>194 ± 31 (160–240)</td>
</tr>
<tr>
<td>C. Macrophages incubated with supernatants of B16 melanoma cultures and normal rat lymphocytes</td>
<td>185 ± 30 (148–226)</td>
<td>186 ± 36 (134–270)</td>
</tr>
<tr>
<td>D. Macrophages incubated with supernatants of B16 melanoma cultures and rat lymphocytes sensitized to B16 melanoma</td>
<td>74 ± 30 (34–118)</td>
<td>180 ± 42 (146–230)</td>
</tr>
</tbody>
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No. of pulmonary metastasesa macrophages were injected

* Six mice/group. Pulmonary metastases were counted 14 days post-i.v. injection of macrophages with the aid of a dissecting microscope.
† 200,000 viable macrophages/mouse.
‡ 2,000,000 viable macrophages/mouse.
§ B16 melanoma line F-11.
Mean ± S.D.
Numbers in parentheses represent range.
The average number of pulmonary metastases differed significantly. Group d vs. Group a p < 0.005.

Table 2

<table>
<thead>
<tr>
<th>Macrophage treatment in vitro*</th>
<th>No. of pulmonary metastasesa</th>
<th>Day 14 †</th>
<th>Day 21 †</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Tumor cells alone</td>
<td>14 ± 6</td>
<td>28 ± 5</td>
<td></td>
</tr>
<tr>
<td>B. Macrophages alone (no tumor cells)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>C. Macrophages incubated with supernatants of B16 melanoma cultures</td>
<td>12 ± 5</td>
<td>29 ± 8</td>
<td></td>
</tr>
<tr>
<td>D. Macrophages incubated with supernatants of B16 melanoma cultures and rat lymphocytes sensitized to a.c. 15091</td>
<td>9 ± 2</td>
<td>16 ± 3</td>
<td></td>
</tr>
<tr>
<td>E. Macrophages incubated with supernatants of B16 melanoma cultures and rat lymphocytes sensitized to C57BL/6 lymphocytes</td>
<td>10 ± 2</td>
<td>12 ± 4</td>
<td></td>
</tr>
<tr>
<td>F. Macrophages incubated with supernatants of B16 melanoma cultures and rat lymphocytes sensitized to B16 melanoma</td>
<td>6 ± 3</td>
<td>4 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

* 500,000 macrophages/mouse.
§ Six mice/group.
† Pulmonary metastases were counted on Days 14 or 21 post-i.v. macrophage injection.
‡ B16 melanoma line F-3
The average number of pulmonary metastases differed significantly. F vs. A p < 0.001; F vs. C p < 0.001; F vs. D p < 0.005; F vs. E p < 0.01.

In our experiments, we had used 2 B16 melanoma tumor lines selected in our laboratory. Line F-3 is a low metastasis producer, while line F-11 produces a relatively high number of pulmonary metastases following i.v. injection (7). Although the number of pulmonary metastases varied from one experiment to the other, the results were very similar and the trend was identical. The data demonstrated the following: (a) the dose of macrophages injected i.v. (200,000 or 500,000 viable macrophages) did not change the direction of the inhibitory effects on the outcome of pulmonary metastases; (b) macrophages injected i.p. into mice bearing pulmonary metastases had no inhibitory ef-

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Effects although their number was 10 times higher than those injected i.v. (Table 1); (c) macrophages cultured in vitro with supernatants of either B16 melanoma alone or normal nonsensitized rat lymphocytes and the B16 melanoma had no inhibitory effects on the established metastases; (d) macrophages treated with supernatants obtained from in vitro cultures of B16 melanoma and rat lymphocytes sensitized in vivo to a.c. 15091 (nonspecific sensitization) had demonstrated some inhibitory activity on metastasis formation (p < 0.01; Table 2); (e) macrophages treated with supernatants obtained from in vitro cultures of B16 melanoma and rat lymphocytes sensitized in vivo to C57BL/6 normal lymphocytes had demonstrated an increased significant inhibitory activity to the formation of metastases (p < 0.01); (f) the most significant inhibition of pulmonary metastasis formation was observed with macrophages treated in vitro with supernatants of B16 melanoma cultures incubated with rat lymphocytes sensitized to the B16 melanoma in vivo. The difference between pulmonary metastases of control animals and those given injections of macrophages “specifically activated” was highly significant (p < 0.001); (g) moreover, with a higher ratio of macrophages to tumor cells (Table 2), the decrease in pulmonary metastases was even greater; (h) the decrease in pulmonary metastases by syngeneic macrophages was related to their treatment in vitro, dose, and the duration of their activity in vivo. The latter is noted in Table 2. In this experiment mice were killed either 14 or 21 days following the i.v. injection of the in vitro-activated macrophages. It appeared that, at Day 21 post-macrophage injection in all mice but those given injections of macrophages activated by supernatants of rat lymphocytes sensitized in vivo to B16 melanoma, the number of pulmonary metastases had increased. On the other hand, mice in Group F actually had fewer lung metastases on Day 21 posttreatment than on Day 14 posttreatment; finally (i) there was a demonstrable difference in size of pulmonary metastases between mice given injections of Group F macrophages (1 mm or less in diameter) and mice in all other groups (2 to 4 mm in diameter).

**DISCUSSION**

Stimulated lymphocytes release a large number of biologically active mediators that participate in the phenomenon of cellular reactivity in vivo. In vitro investigations of these mediators strongly suggest that some are chemotactic to macrophages, preventing their migration out of the area and, in some cases, activating the macrophages and rendering them cytotoxic (for review, see Ref. 2). In vitro, normal mouse macrophages could be rendered cytotoxic by several closely related methods. In a syngeneic system, supernatants collected from cultures of sensitized spleen cells and tumor cells (S) as well as thymocytes from sensitized animals (10) when incubated with normal macrophages could render them cytotoxic. Specifically, thymocytes from sensitized mice released a macrophage-activating factor when cultured with the target cells (10, 13). In an allogeneic system, the factor that renders normal macrophages cytotoxic has been reported to be a product of thymus-dependent lymphocytes. Once released, it could render allogeneic as well as syngeneic macrophages cytotoxic (13).

Our experiments dealt with activation of macrophages by a factor released by non-glass-adhering xenogeneic lymphocytes obtained from normal and/or sensitized rats. It appeared that only the lymphocytes from rats that were sensitized in vivo to the B16 melanoma and to a lesser degree to the C57BL/6 lymphocytes and then cultured with the tumor target in vitro released a factor capable of activating the mouse macrophages from tumor-bearing mice. This finding agrees with the earlier published reports dealing with syngeneic or allogeneic activation of macrophages and confirms the observation that the mechanism responsible for the release of lymphocytic mediators is specific in nature. However, once the activating factor is released it can render syngeneic, allogeneic and, in our system, xenogeneic macrophages cytotoxic to the tumor target cells in vivo and in vitro (unpublished data).

Previous studies of cytotoxic macrophages concentrated on their behavior in vitro. In the present investigation we utilized an *in vivo* experimental metastasis assay to demonstrate the ability of activated macrophages to destroy the specific tumor target cells. In our earlier studies of the quantitative analysis of cancer metastasis (6), we demonstrated that the majority of circulating tumor cells rapidly die; but tumor cells which by 1 to 2 days post-i.v. injection are established in the lung parenchyma will continue to grow progressively and kill the recipient animal (6, 7). In the present experiment we studied the *in vivo* inhibitory effects of *in vitro*-activated syngeneic macrophages on established pulmonary metastases (which untreated would have progressed to kill the recipient animal). The macrophages obtained from the mice bearing the B16 melanoma s.c. were either not cytotoxic *in vivo* or not sufficiently effective as seen by the constant and rapid progression of tumor growth leading to death of the host. It is therefore most significant that these macrophages could be rendered cytotoxic by the xenogeneic lymphocyte supernatants. Indeed, this approach to macrophage activation could be a method of overcoming or even bypassing the possible defect in the syngeneic immune mechanism to the neoplasm.

Many questions remain unanswered: (a) Will xenogeneic lymphocytes sensitized *in vivo* to one mouse tumor and then incubated *in vitro* with a different tumor release any activating factors? (b) Is the activation of syngeneic macrophages a specific phenomenon? (c) Do cytotoxic macrophages kill tumor selectively? Our preliminary unpublished data agree with others (11, 12) that cytotoxic macrophages do not kill normal syngeneic cells. (d) What is the optimal ratio of activated macrophages to tumor cells to be injected *in vivo* to bring about complete inhibition of metastases? (e) Could activated macrophages inhibit metastatic foci that are more advanced than those in the current study? These and other related questions are now under investigation.

Our current data support the experimental findings that cytotoxic macrophages may occupy a major role in the defense mechanism to neoplasia. Moreover, the i.v. injec-
tion of syngeneic macrophages following their treatment \textit{in vitro} with supernatants obtained from sensitized xenogeneic lymphocytes could provide an approach to possible therapy.

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