Immune Stimulation-Inhibition of Experimental Cancer Metastasis

Isaiah J. Fidler

Department of Pathology, School of Dental Medicine and Center for Oral Health Research, University of Pennsylvania, Philadelphia, Pennsylvania, 19174

SUMMARY

The interaction of normal, tumor-bearing, nonspecifically sensitized, and immunized syngeneic lymphocytes and the B16 melanoma was tested in vivo by experimental pulmonary metastases.

Various numbers of lymphocytes were mixed and incubated with the tumor cells for 90 min on a rotating platform, and then the mixtures were injected i.v. into C57BL/6 mice. Normal, tumor-bearing, and nonspecifically sensitized lymphocytes mixed with tumor cells significantly increased the incidence of experimental metastasis. On the other hand, a high number (5000:1) of immunized syngeneic lymphocytes mixed with the B16 melanoma cells brought about a dramatic decrease in the incidence of subsequent pulmonary metastases. Following the in vitro incubation of tumor cells with syngeneic lymphocytes, clumping of tumor cells was observed. The relative importance of this clumping phenomenon to the outcome of experimental metastasis is discussed.

Mice that were either immunized against the B16 melanoma or thymectomized and X-irradiated demonstrated a significant decrease in incidence of experimental pulmonary metastasis following i.v. injection of tumor cells as compared to normal, thymectomized, or X-irradiated mice. The decrease in pulmonary metastases in thymectomized X-irradiated mice was completely reversible with i.v. injection of \(1 \times 10^7\) syngeneic tumor-bearing lymphocytes administered 24 hr prior to tumor cell injection. On the other hand, administration of \(1 \times 10^6\) syngeneic tumor-bearing lymphocytes brought about a significant decrease in the incidence of pulmonary metastases in all experimental groups. These results further support the hypothesis and work of Prehn and our earlier reports, that the immune response may have a dual role in its relationship to the development, progression, and perhaps the spread of cancer.

INTRODUCTION

Prehn has advanced the theory that weakly antigenic tumors or a weak incipient immune response may bring about a cellular immune reactivity that initially is stimulatory to the tumor cell growth. Once the immune response is active, inhibition to tumor growth could occur (27-29). Subsequently, Prehn (28) demonstrated that low ratios of specifically sensitized spleen cells mixed with a constant number of tumor cells and then injected s.c. into immunosuppressed mice actually aided the growth of the tumor.

We have recently reported the results of the interaction of normal sensitized and concanavalin A-stimulated syngeneic, allogeneic, and/or xenogeneic lymphocytes with the B16 melanoma, C57BL/6, and/or A mouse embryo cells in an in vitro colony inhibition-stimulation system. Specifically sensitized lymphocytes at ratios up to \(1000:1\) repeatedly and significantly enhanced the growth of the target cells. At higher lymphocyte ratios, target cell inhibition occurred. The mechanism by which sensitized lymphocytes enhanced tumor growth appeared to be by direct interaction (8). Similarly, in studies with spontaneous canine tumors, low doses of lymphocytes in the absence of autologous serum brought about stimulation of target tumor cells in vitro. Furthermore, serum from tumor-bearing dogs while blocking lymphocyte-mediated cytotoxicity actually potentiated the tumor growth seen with low numbers \((100:1)\) of sensitized lymphocytes (10).

The present report concerns the effects of in vitro and in vivo interaction of immune cells and tumor cells on the outcome of experimental pulmonary metastases. The studies were performed to gain information relevant to several questions. First, could the outcome of experimental metastasis be influenced by the interaction in vitro of normal, tumor-bearing lymphocytes, nonspecifically sensitized and/or immunized lymphocytes, and the B16 melanoma tumor cells? Second, could immune stimulation-inhibition by a varying ratio of lymphocytes to tumor cells be demonstrated in an experimental metastasis system? Third, could the outcome of experimental metastasis be influenced by in vivo manipulation of recipient immune system?

MATERIALS AND METHODS

The cell types used in the present studies are designated as follows.

Normal Lymphocytes. Lymphocytes obtained from lymph nodes and spleens of control C57BL/6 mice.

Tumor-bearing Lymphocytes. Lymphocytes obtained from lymph nodes and spleens of C57BL/6 mice bearing the B16 melanoma s.c.

Sensitized Lymphocytes. Lymphocytes obtained from lymph nodes and spleens of C57BL/6 mice that were given i.p. injections of xenogeneic spleen cells.

Immunized Lymphocytes. Lymphocytes obtained from lymph nodes and spleens of C57BL/6 mice immunized against the B16 melanoma.

1 Supported by USPHS Research Grants CA 12456 and DE 02623.

Received August 24, 1973; accepted November 19, 1973.
Isaiah J. Fidler

Animals. Inbred C57BL/6 mice were obtained from The Jackson Laboratories, Bar Harbor, Maine. Fischer 344 rats were supplied by Microbiological Associates, Inc., Bethesda, Md.

Tumor. The transplantable B16 melanoma, originating in C57BL/6 mice, was obtained originally from The Jackson Laboratories.

Immunizations of C57BL/6 Mice against B16 Melanoma. C57BL/6 mice were given s.c. injections of $1 \times 10^8$ melanoma tumor cells that had been exposed to 12,000 R X-irradiation and mixed with complete Freund's adjuvant (1). Mice were given injections 3 times at 2-week intervals and then challenged s.c. with 50,000 viable unirradiated B16 cells. An alternate method of immunization which yielded better results was as follows. B16 melanoma was grown i.p. in C57BL/6 mice. Cells were harvested and pressurized through a stainless steel 50 mesh sieve into HBSS. Tumor cells were then freeze-thawed 3 times as described previously (22). Again, mice were given s.c. injections of $1 \times 10^8$ freeze-thawed cells 3 times at 2-week intervals and then challenged s.c. with 50,000 viable B16 melanoma cells.

In either procedure, only the mice that rejected the above normally lethal tumor inoculum were designated as immunized animals, and their spleen and lymph nodes were collected for our studies.

Sensitization in Vivo of C57BL/6 Mice to Fischer Rat Transplantation Antigens. C57BL/6 mice 8 to 10 weeks old were given s.c. injections of $1 \times 10^8$ nucleated Fischer spleen cells in complete Freund's adjuvant. One week later the animals were given i.p. injections of $1 \times 10^7$ nucleated Fischer spleen cells. Mice were killed 7 to 10 days following the 2nd injection, and their spleens and lymph nodes were collected.

Sensitization of C57BL/6 Mice to B16 Melanoma. C57BL/6 mice 6 to 8 weeks old were given s.c. injections of 100,000 viable B16 cells. Two to 3 weeks later when the tumors were between 10 to 30 mm in size the animals were killed. Their lymph node and spleen lymphocytes were collected and designated as tumor-bearing lymphocytes.

B16 Melanoma Cultures. The transplantable B16 melanoma was adapted to growth in tissue culture as described previously (5, 7, 8). Stock cultures were maintained on glass in Eagle's minimum essential medium, supplemented with 10% fetal calf serum, vitamin solution, sodium pyruvate, nonessential amino acids, penicillin-streptomycin, and L-glutamine. This medium is designated as complete minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.). The cells were cultured in a humidified 37° incubator containing 5% CO₂.

Preparation of Normal and Sensitized Lymphocytes. Axillary, cervical, and mesenteric lymph nodes and spleens were collected aseptically from normal or sensitized animals, placed in HBSS, and forced through a wire mesh sieve. The resulting suspensions were filtered through a glass wool column and centrifuged, and the cellular pellets were resuspended in complete minimum essential medium.

Procedure for Study of Experimental Metastasis in Vivo in Normal C57BL/6 Mice. B16 melanoma cells grown in vitro were harvested during their exponential growth phase by 1-min trypsinization (0.25% trypsin:EDTA solution), washed twice, and resuspended in HBSS. The number of single viable tumor cells was determined. Spleen and lymph node lymphocytes were collected from normal, tumor-bearing (C57BL/6 x B16 melanoma). Nonspecifically sensitized (C57BL/6 x Fischer rat) and immunized mice were prepared as described above. Following all viability tests the number of viable tumor cells was adjusted to contain 100,000 cells/ml HBSS. The addition of 1 ml lymphocyte suspension to the tumor cells yielded a final dilution of 50,000 tumor cells per ml and various numbers of lymphocytes. Specifically, the ratios of lymphocytes to tumor cells were 100:1, 250:1, 500:1, 1000:1, 2500:1, and 5000:1.

In addition, control groups of tumor cells alone or lymphocytes alone were included. These mixtures were placed into test tubes on a rotating platform and incubated at 25° for 90 min. This procedure enhances tumor cell-lymphocyte direct interaction as was described previously (8-10).

Following this incubation, tumor cells alone (no lymphocytes) and the various mixtures were injected i.v. into the tail vein of C57BL/6 mice. Inoculum volume per mouse for all experiments was 0.2 ml. Mice were killed 14 days after tumor cell-lymphocyte injection, and the number of resultant pulmonary metastases was determined, with the aid of a dissecting microscope, by 2 independent observers.

Procedure for Study of Experimental Metastasis in Immune-manipulated Mice. In this study all C57BL/6 mice received a 0.2-ml inoculum dose containing 25,000 viable B16 melanoma cells. The syngeneic recipients have been divided into 5 experimental groups: Group A, control mice; Group B, mice thymectomized 5 weeks prior to experiment (at 5 weeks of age); Group C, mice thymectomized 5 weeks prior to experiment and X-irradiated (400 R) 4 weeks prior to experiment; Group D, mice X-irradiated (400 R) 4 weeks prior to experiment, and Group E, mice immunized to the B16 melanoma (as described above). All animals were killed 14 days after i.v. tumor cell injection; at this time their pulmonary metastases were counted, with the aid of a dissecting microscope, by 2 independent observers.

Procedure for Study of Experimental Metastasis in Immune-suppressed C57BL/6 Mice with or without Immune Reconstitution. In this study mice were divided into 3 major groups: Group A, normal control mice; Group B, adult-thymectomized X-irradiated (400 R) mice, and Group C, adult sham-thymectomized X-irradiated (400 R) mice. One day prior to tumor cell injection each group was subdivided into two. The mice in the 1st subgroup were given i.v. injections of $1 \times 10^7$ lymphocytes obtained from C57BL/6 mice bearing B16 melanoma growing s.c. The mice in the 2nd subgroup served as untreated controls. One day later, all animals were given i.v. injections, into the tail vein, of 0.2 ml B16 tumor suspension containing 25,000 viable cells.
Fourteen days later the mice were killed, and their lung metastases were counted with the aid of a dissecting microscope. In a similar study, mice were reconstituted with 1 × 10^6 tumor-bearing C57BL/6 lymphocytes to determine their effect on experimental metastasis.

**X-irradiation.** Whole-body irradiation of mice was accomplished with exposure to 400 R γ-irradiation from a ^137^Cs source emitting at a constant rate of 108 rads/min (Gamma Cell 40, Atomic Energy of Canada, Ltd.) (11).

**Statistical Analysis.** Statistical analysis was carried out by the Student t test.

### RESULTS

The viability of the B16 melanoma cells alone, lymphocytes alone, or the mixtures of tumor cells and lymphocytes, just prior to i.v. injection, was always above 90%. Following *in vitro* incubation of lymphocytes with tumor cells, multiple clumps of tumor cells could be observed microscopically. These clumps ranged from 3 to about 30 cells in size. Most clumps consisted of tumor cells and lymphocytes with the latter adhering to the tumor cell surface (Figs. 1 and 2). It was not clear whether the lymphocytes were directly responsible for aggregating the tumor cells or whether the lymphocytes formed "chains" and tumor cells were trapped passively.

**Relationship of Injected Tumor Cells-Lymphocytes Mixture to Pulmonary Metastases.** Normal, tumor-bearing, nonspecifically sensitized, and immunized lymphocytes were tested for their ability to affect the outcome of experimental metastasis *in vivo*. The findings obtained are shown in Tables 1 to 3. Several experiments were carried out with the B16 melanoma. Lymphocytes from normal and presensitized mice were mixed with the B16 cells at ratios ranging from 100:1 to 5000:1 (lymphocytes to tumor cells). All lymphocytes at low ratios (100:1, 250:1, and 500:1) significantly increased the incidence of subsequent pulmonary metastases (*p* < 0.001). Normal or nonspecifically sensitized C57BL/6 lymphocytes also enhanced metastases in higher doses (2500:1 or 5000:1). When tumor-bearing or immunized C57BL/6 lymphocytes were mixed with the B16 cells at high doses (5000:1), a dramatic decrease in the number of metastases was demonstrable (Table 3). This decrease [1.8 ± 1 (S.D.) metastases] was highly significant as compared to controls (22 ± 6) (*p* < 0.001).

**Relationship of Pulmonary Metastases and Immune Manipulation of Recipient C57BL/6 Mice.** The results of the previous studies demonstrated the enhancing effects of lymphocytes mixed *in vitro* with the B16 cells on experimental metastasis. It was now necessary to investigate whether this phenomenon could be demonstrated by the manipulation of the immune response of recipient animals in order to achieve corresponding effects. The results of this study are shown in Table 4. The data demonstrated that adult thymectomy alone or total body X-irradiation 4 weeks prior to tumor cell injection had no significant effects on the incidence of pulmonary metastases. On the other hand, prior immunization to the tumor resulted in a significant decrease of experimental pulmonary metastases (72 ± 28).

### Table 1

<table>
<thead>
<tr>
<th>No. and type of cells injected i.v.</th>
<th>Av. no. of pulmonary metastases*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 10,000 B16 cells, no lymphocytes</td>
<td>12 ± 6* (7-20)*</td>
</tr>
<tr>
<td>B. 10,000 B16 cells, 100:1 normal C57BL/6 lymphocytes</td>
<td>19 ± 8 (10-31)*</td>
</tr>
<tr>
<td>C. 10,000 B16 cells, 1000:1 normal C57BL/6 lymphocytes</td>
<td>21 ± 6 (9-37)*</td>
</tr>
<tr>
<td>D. 10,000 B16 cells, 100:1 tumor-bearing lymphocytes C57BL/6</td>
<td>19 ± 3 (9-24)*</td>
</tr>
<tr>
<td>E. 10,000 B16 cells, 1000:1 tumor-bearing lymphocytes C57BL/6</td>
<td>23 ± 4 (14-53)*</td>
</tr>
<tr>
<td>F. 10,000 B16 cells, 100:1 C57BL/6 lymphocytes sensitized to Fischer rats</td>
<td>49 ± 10 (32-67)*</td>
</tr>
<tr>
<td>G. 10,000 B16 cells, 100:1 C57BL/6 lymphocytes sensitized to Fischer rats</td>
<td>42 ± 9 (34-80)*</td>
</tr>
<tr>
<td>H. 10,000 B16 cells, 100:1 immunized lymphocytes C57BL/6</td>
<td>37 ± 16 (20-60)*</td>
</tr>
<tr>
<td>I. 10,000 B16 cells, 1000:1 immunized lymphocytes C57BL/6</td>
<td>39 ± 8 (22-58)*</td>
</tr>
</tbody>
</table>

*Ten mice/group. Pulmonary metastases were counted 14 days after i.v. injection with the aid of a dissecting microscope.

*Mean ± S.D.

*Group A differed from all other groups (*p* < 0.005).

*Glass wool column-filtered lymph node and spleen lymphocytes from normal C57BL/6 mice.

*Glass wool column-filtered lymph node and spleen lymphocytes from C57BL/6 mice with B16 melanoma growing s.c.

*Glass wool column-filtered lymph node and spleen lymphocytes from C57BL/6 mice immunized against B16 melanoma.

### Table 2

<table>
<thead>
<tr>
<th>No. and type of cells injected i.v.</th>
<th>Av. no. of pulmonary metastases*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 10,000 B16 cells, no lymphocytes</td>
<td>6 ± 1 (2-8)*</td>
</tr>
<tr>
<td>B. 10,000 B16 cells, 250:1 normal C57BL/6 lymphocytes</td>
<td>50 ± 18 (15-96)*</td>
</tr>
<tr>
<td>C. 10,000 B16 cells, 250:1 normal C57BL/6 lymphocytes</td>
<td>56 ± 8 (11-140)*</td>
</tr>
<tr>
<td>D. 10,000 B16 cells, 250:1 C57BL/6 tumor-bearing lymphocytes</td>
<td>75 ± 12 (40-135)*</td>
</tr>
<tr>
<td>E. 10,000 B16 cells, 250:1 C57BL/6 tumor-bearing lymphocytes</td>
<td>7 ± 2 (3-18)*</td>
</tr>
<tr>
<td>F. 10,000 B16 cells, 250:1 C57BL/6 lymphocytes sensitized to Fischer rats</td>
<td>30 ± 7 (16-74)*</td>
</tr>
<tr>
<td>G. 10,000 B16 cells, 250:1 C57BL/6 lymphocytes sensitized to Fischer rats</td>
<td>34 ± 20 (16-65)*</td>
</tr>
</tbody>
</table>

*Eight mice/group. Pulmonary metastases were counted 14 days after i.v. injection with the aid of a dissecting microscope.

*Mean ± S.D.

*Glass wool column-filtered lymph node and spleen lymphocytes from normal C57BL/6 mice.

*Group A vs. B, C, D, F, G, *p* < 0.005; Group D vs. E, *p* < 0.001.

*Glass wool column-filtered lymph node and spleen lymphocytes from C57BL/6 mice bearing B16 melanoma.

*Glass wool column-filtered lymph node and spleen lymphocytes from C57BL/6 mice sensitized *in vivo* to Fischer rat.
Table 3
Number of pulmonary metastases in lungs of C57BL/6 mice following i.v. injection of 10,000 viable B16 melanoma cells mixed with or without normal, tumor-bearing, or immunized lymphocytes

<table>
<thead>
<tr>
<th>No. and type of cells injected i.v.</th>
<th>Av. no. of pulmonary metastases*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 10,000 B16 cells, no lymphocytes</td>
<td>22 ± 6* (9-39)*</td>
</tr>
<tr>
<td>B. 10,000 B16 cells, 500:1 normal</td>
<td>91 ± 20 (56-121)*</td>
</tr>
<tr>
<td>C. 10,000 B16 cells, 5000:1 normal</td>
<td>75 ± 28 (32-102)*</td>
</tr>
<tr>
<td>D. 10,000 B16 cells, 500:1 tumor-bearing</td>
<td>100 ± 27 (58-153)*</td>
</tr>
<tr>
<td>E. 10,000 B16 cells, 5000:1 tumor-bearing</td>
<td>12 ± 2 (6-15)*</td>
</tr>
<tr>
<td>F. 10,000 B16 cells, 500:1 immunized</td>
<td>60 ± 12 (42-92)*</td>
</tr>
<tr>
<td>G. 10,000 B16 cells, 5000:1 immunized</td>
<td>1.8 ± 1 (0-4)*</td>
</tr>
</tbody>
</table>

* Twelve mice/group. Pulmonary metastases were counted 14 days after i.v. injection with the aid of a dissecting microscope.
\* Mean ± S.D.
\* Group A vs. B, C, D, p < 0.001; Group A vs. G, p < 0.001.
\* Glass wool column-filtered lymph node and spleen lymphocytes from normal mice.
\* Glass wool column-filtered lymph node and spleen lymphocytes from C57BL/6 mice with B16 melanoma growing s.c.
\* Glass wool column-filtered lymph node and spleen lymphocytes from mice immunized against the B16 melanoma.

Table 4
Number of pulmonary metastases in normal and immunomanipulated C57BL/6 mice following i.v. injection of 25,000 viable B16 melanoma cells

<table>
<thead>
<tr>
<th>Group of C57BL/6 mice given i.v. injections</th>
<th>Av. no. of pulmonary metastases*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Control-normal mice</td>
<td>199 ± 50* (121-251)</td>
</tr>
<tr>
<td>B. Thymectomized mice*</td>
<td>212 ± 52 (130-270)</td>
</tr>
<tr>
<td>C. Thymectomized-X-irradiated*</td>
<td>62 ± 27 (16-88)*</td>
</tr>
<tr>
<td>D. X-irradiated mice*</td>
<td>240 ± 20 (140-290)</td>
</tr>
<tr>
<td>E. Immunized mice*</td>
<td>72 ± 28 (15-130)*</td>
</tr>
</tbody>
</table>

* Twelve mice/group, pulmonary metastases counted with the aid of a dissecting microscope, 14 days following i.v. tumor cell injection.
\* Mean ± S.D.
\* Thymectomized at 4 to 5 weeks of age, 5 weeks prior to tumor cell injection.
\* Total-body irradiation of 400 R 1 week postthymectomy, 4 weeks prior to tumor cell injection.
\* The difference in number of metastases was significantly decreased as compared to the controls (p < 0.001).
\* C57BL/6 mice immunized to B16 melanoma.

as compared to control mice (199 ± 50) (p < 0.001). Moreover, adult thymectomy followed 1 week later by 400 R total-body irradiation resulted in a significant decrease of pulmonary metastases (62 ± 27) as compared to controls (p < 0.001). The results of this experiment demonstrated that a decrease in incidence of pulmonary metastases occurred in mice that were either immunosuppressed by adult thymectomy and X-irradiation or those that were immunized to the B16 melanoma. It became apparent that further clarification of the possible mechanism responsible for this phenomenon was necessary. Adult mice (4 to 5 weeks) were either thymectomized or sham-thymectomized and 1 week later were X-irradiated with 400 R. Four weeks later, these 2 groups of mice and normal controls were subdivided into 2 treatment groups consisting of 8 or 12 mice each. One subgroup remained untreated, and in the other each mouse received an i.v. injection of 1 x 10^7 lymphocytes obtained from C57BL/6 mice bearing s.c. B16 melanoma (tumor-bearing lymphocytes). Twenty-four hr later, all mice were given i.v. injections of 10,000 viable B16 cells. This ratio of lymphocytes to tumor cells was 1,000:1. Fourteen days later the mice were killed and their lung metastases were counted. The results of this study are summarized in Table 5. Again, thymectomy-X-irradiation brought about a significant decrease in the number of pulmonary metastases (18 ± 3) as compared to the controls (36 ± 5) (p < 0.005). On the other hand, sham-thymectomy X-irradiation had no significant effects on the outcome of pulmonary metastases.

All mice that were given injections of 1 x 10^7 C57BL/6 tumor-bearing lymphocytes (spleen and lymph nodes) demonstrated a significant increase in the incidence of pulmonary metastases as compared to their unconstituted respective controls. The most striking result was observed in mice thymectomized and X-irradiated where injection of tumor-bearing syngeneic lymphocytes 24 hr prior to the tumor cell injection dramatically increased the incidence of pulmonary metastases from 18 ± 3 to 75 ± 10 nodules (p < 0.001). It was thus concluded that the administration of sensitized syngeneic lymphocytes completely reversed the decrease in metastases seen in the immunosuppressed mice. In the final study, the mice were again divided into 2 major subgroups. However, "reconstituted" mice were given i.v. injections of 1 x 10^7 tumor-bearing lymphocytes, 24 hr prior to the B16 injection. The ratio of lymphocytes to normal, tumor-bearing, or immunized lymphocytes was 1,000:1. Fourteen days after the i.v. injection, all mice were killed and their lung metastases were counted. The results of this study are summarized in Table 6. The difference in number of metastases was significantly decreased as compared to the controls (p < 0.001).

Table 5
Number of pulmonary metastases in control, thymectomized-X-irradiated, and sham-thymectomized-X-irradiated C57BL/6 mice either untreated or treated by i.v. injection of 1 x 10^7 tumor-bearing C57BL/6 lymphocytes 24 hr prior to i.v. injection of 10,000 viable B16 melanoma cells

<table>
<thead>
<tr>
<th>Group of C57BL/6 mice given i.v. injections</th>
<th>Av. no. of pulmonary metastases*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Normal controls</td>
<td>36 ± 5* (32-45)*</td>
</tr>
<tr>
<td>B. Thymectomized-X-irradiated*</td>
<td>18 ± 3 (10-25)*</td>
</tr>
<tr>
<td>C. Sham-thymectomized-X-irradiated*</td>
<td>30 ± 11 (17-41)*</td>
</tr>
</tbody>
</table>

* Eight mice/group; pulmonary metastases were counted 14 days after i.v. injection with the aid of a dissecting microscope.
\* Mean ± S.D.
\* The differences in pulmonary metastases between untreated mice and lymphocyte-treated mice were significant (p < 0.01).
\* Thymectomized at 4 to 5 weeks of age 5 weeks prior to tumor cell injection; 400 R total-body irradiation 4 weeks prior to tumor cell injection.
\* The decrease in numbers of pulmonary metastases as compared to normal mice was significant (p < 0.001).
The difference was significant (p < 0.005). No discernible
summarized in Table 6. Normal animals had a higher
tumor cells in this study was high, 10,000:1. The results are
incidence of pulmonary metastases (164 ± 12) as compared
to the thymectomized, X-irradiated mice (93 ± 7), and this
difference was significant (p < 0.005). No discernible
difference was demonstrable between the normal mice and
those thymectomized 4 weeks earlier. In all mice receiving 1
× 10⁸ tumor-bearing lymphocytes 24 hr prior to tumor cell
injection, a decrease in incidence of pulmonary metastasis
was observed. Thus a ratio of 10,000:1 for lymphocytes to
tumor cell had inhibited rather than stimulated the forma-
tion of experimental pulmonary metastasis.

DISCUSSION
Cancer metastasis is a complicated biological phenome-
non which is influenced by a multitude of factors. The
simple presence of tumor cells in a patient’s blood does not
constitute a metastasis since most emboli do not survive to
yield secondary growths (5, 6, 15, 33). Recent quantitative
studies of the mechanisms of metastasis demonstrated that
only about 0.1% of injected circulating tumor cells survived
to form secondary growths. There are several difficulties
that must be overcome when an experimental study of
cancer metastasis is utilized. In earlier studies we dealt with
some of these difficulties and demonstrated the following:
(a) the number of resultant experimental pulmonary metas-
tases is proportional to the (dose) number of viable tumor
cells injected i.v. but is influenced not just by the viability of
tumor emboli but also by the total number of circulating
emboli, since dead tumor cells and even syngeneic embryo
cells when injected simultaneously with live tumor cells
greatly increased the incidence of lung metastasis; (b) tumor
cells in small clumps (4 to 5 cells) are arrested and survive
better to yield subsequent metastases than the same cells in
a single-cell suspension (6); (c) the survival of circulating
malignant emboli is not a random phenomenon, but rather
these tumor cells possess unique qualities which allow for
their survival. This was concluded from our studies of clonal
selection of tumor cells from successive lung metastases
which yielded tumor cell lines better capable of survival in
vivo to form secondary growths (7). Our studies of the
mechanisms responsible for enhancement of metastasis in
mice receiving 450 R total-body X-irradiation (12) or given
corticosteroid injections (11), indicated that an apparent
increase in the initial arrest of tumour cells in the capillary
bed of an organ (i.e., lungs) was the most important factor
contributing to the enhancement of subsequent pulmonary
metastases. Similarly, Gasic et al. (18) reported that platelet
aggregation contributed directly to the outcome of metasta-
sis. The proposed mechanism could have been an increase in
the arrest of tumor emboli in microcirculation or, alterna-
tively, released platelet contents could have enhanced
metastasis by increasing tumor cell motility and/or vascular
permeability.

For many years investigators suspected that some cir-
culating tumor cells may be destroyed by specific host
immune mechanisms but the evidence for such a hypothesis
is still lacking. Certainly, some studies actually contradicted
the view that a patient’s resistance to tumor is related to the
activity of its reticuloendothelial system (13). Borberg et al.
(2) reported that i.v. injection of lymphocytes from immu-
nized syngeneic or allogeneic mice or even from sheep
brought about inhibition of tumor grafts in mice. On the
other hand, Fisher et al. (17) did not find the results
following injection of sensitized lymphocytes to be inhibi-
tory to tumor transplant growth. It would appear that the
outcome of such experiments may be related directly to the
number of lymphocytes transferred (i.e., a high number of
lymphocytes is necessary to achieve tumor growth inhibition
in vivo).

Many immunological and nonimmunological functions
have been attributed to the lymphoreticular system in
general and to the small lymphocyte in particular. The data
concerning the “trephocytic” function of lymphocytes serv-
ing as a possible source of essential growth substances for
various organs were reported by Carrell in 1922 (3). Indeed,
several investigators have suggested that the lymphoreticu-
lar system regulates the growth of other normal tissue (for
review, see Ref. 29). Stimulation of tumor and normal cell
growth in vitro was clearly demonstrated in our recent
studies dealing with mouse tumors and transplantation
systems (8, 9) and with spontaneous dog tumors of various
histological types (10). In the latter we found that autolo-
gous serum was able to block the lymphocyte-mediated
cytotoxicity in vitro but, in addition, the serum actually
potentiated tumor growth stimulation mediated by a low
number of lymphocytes (10). These findings agreed closely
with earlier reports by Prehn (28), Medina and Heppner
(26), and Heppner et al. (20).

In the present studies the incidence of experimental
metastasis was significantly increased when the tumor cells
were preincubated in vitro with various doses of normal,
nonspecifically sensitized, specifically sensitized, and/or
immunized syngeneic lymphocytes. On the other hand,
tumor cell incubation with high doses (5000:1) of lympho-

### Table 6

Table 6 Number of pulmonary metastases in control, thymectomized-X-
irradiated, and sham-thymectomized-X-irradiated C57BL/6 mice not
treated or treated by i.v. injection of 1 x 10⁸ tumor-bearing C57BL/6
lymphocytes 24 hr prior to i.v. injection of 20,000 viable B16 melanoma
cells.

<table>
<thead>
<tr>
<th>Group of C57BL/6 mice given i.v. injections</th>
<th>Av. no. of pulmonary metastases¹</th>
<th>Lymphocyte-treated²</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Normal controls</td>
<td>164 ± 12 (115-300)</td>
<td>97 ± 8 (38-129)</td>
</tr>
<tr>
<td>B. Thymectomized-X-irradiated</td>
<td>93 ± 7 (56-137)</td>
<td>72 ± 6 (40-117)</td>
</tr>
<tr>
<td>C. Sham-thymectomized-X-irradiated</td>
<td>152 ± 10 (93-330)</td>
<td>38 ± 5 (15-60)</td>
</tr>
</tbody>
</table>

¹ Twelve mice/group, pulmonary metastases were counted 14 days after i.v. injection with the aid of a dissecting microscope.
² Lymphocytes (1 x 10⁸/mouse) from C57BL/6 mice bearing B16 melanoma injected i.v. 24 hr prior to tumor cell injection.
³ The differences in pulmonary metastases between untreated mice and lymphocyte-treated mice were significant (p < 0.005).
⁴ Thymectomized at 4 to 5 weeks of age 5 weeks prior to tumor cell injection; 400 R total-body irradiation 4 weeks prior to tumor cell injection.

MARCH 1974 495
cytes from C57BL/6 mice immunized to the B16 melanoma brought about a dramatic decrease in the number of pulmonary metastases. The results agree closely with the work by Prehn (28), which demonstrated that low numbers of sensitized syngeneic lymphocytes mixed with a constant number of tumor cells and then injected into thymectomized X-irradiated mice actually enhanced in vivo tumor growth.

When an animal is preimmunized and then challenged with live tumor cells, an acceleration of tumor growth can sometimes be observed. The phenomenon has been termed tumor enhancement and can be transferred by passage of immune lymphocytes (23). Generally, the mechanism responsible for enhancement of tumor growth in vivo is thought to be related to presence of the so-called blocking antibodies (19–21, 30, 31). These serum factors, which interfere with cellular-mediated cytotoxicity, are presumed to be either antibodies, antigen-antibody complexes, antigen alone, or even nonimmunological (19). In our present experiments, as well as in earlier work on immune stimulation in vitro, we have utilized glass wool-filtered lymphocytes and carried out the tests for a relatively short time to rule out that the accelerated tumor growth was due to blocking antibodies. Presence of blocking antibodies in serum has been correlated to enhancement of metastasis in an experimental system. Duff et al. (4) have reported that injection with HSV types I and II and with simian virus 40 failed to induce immunity in weanling golden hamsters to transformed hamster cells. On the other hand, such prior immunization with HSV type I resulted in a marked enhancement of metastatic tumors. The authors proposed that blocking antibodies to the HSV type I prevented cellular-mediated cytotoxicity which brought about enhancement of metastases (4).

In the present studies we observed that either specific immunization or thymectomy-X-irradiation of recipient C57BL/6 mice brought about a decrease in the incidence of experimental metastasis. Thymectomy alone or X-irradiation alone did not affect the outcome. Indeed, total-body X-irradiation should enhance experimental metastasis (12). These results agreed with an earlier report by Fisher and Fisher (14) that neonatal thymectomy resulted in a decrease rather than an increase in the incidence of metastases. At the same time, these thymectomized rats were unable to reject skin homografts. Further evidence for the possible role that lymphocytes may play in the process of metastasis was obtained from the present study in which mice were given injections of \(1 \times 10^7\) syngeneic lymphocytes obtained from animals bearing the B16 tumor. Unreconstituted, thymectomized, X-irradiated mice had a significant decrease in the number of their lung metastases as compared to unreconstituted, sham-thymectomized-X-irradiated, or normal mice. However, once the mice are injected with \(1 \times 10^7\) tumor-bearing lymphocytes, the pulmonary metastases of the thymectomized X-irradiated mice increased dramatically and did not differ from the number of metastases in the control mice. Specifically, tumor-bearing lymphocytes at the ratio of about 1,000:1 \((1 \times 10^7\) lymphocytes: \(1 \times 10^4\) B16 cells) injected 24 hr prior to tumor cell injection were able to reverse the decrease in pulmonary metastases observed in the immunosuppressed mice. On the other hand, an injection of \(1 \times 10^6\) tumor-bearing lymphocytes (10,000:1) significantly decreased the incidence of pulmonary metastases in all test groups. We thus conclude that the relative number of tumor-bearing lymphocytes injected prior to i.v. injection of tumor cells could increase or decrease the subsequent incidence of pulmonary metastases. This observation agrees with the conclusions of Borberg et al. (2) as discussed above. What is the mechanism by which a small number of normal, tumor-bearing or immunized lymphocytes increase the incidence of metastases while a high dose of immunized lymphocytes dramatically inhibits their formation? In our earlier in vitro studies of immunostimulation-inhibition of tumor growth, we concluded that the phenomenon depended on a direct lymphocyte to target cell interaction (9). Similar conclusions were reported by MacPherson and Pilch (25). Lymphocyte-mediated cytolysis is known to be a complex phenomenon. The initial process involves direct contact and binding. This binding has been found to be dependent on the presence of magnesium, with the extent of binding related to temperature as well as pH. Lymphocyte-tumor cell binding can take place in the absence of serum; however, serum factors seem to be important for cytolysis to occur (32).

In our present studies we observed that, following the in vitro incubation of the B16 melanoma cells with the various syngeneic lymphocytes, clumps consisting of tumor cells and lymphocytes were formed. At present it is not clear whether this clumping phenomenon was immunologically specific or not. It is possible that the syngeneic lymphocytes bound to the surface of 1 or more tumor cells, thus bridging between them. Alternatively, lymphocytes could aggregate and simply trap tumor cells among them. This particular question is currently under investigation with electron microscope techniques. Whatever the mechanism may be, our earlier studies of the mechanisms of metastasis (6) clearly demonstrated that clumped tumor emboli survived better to yield metastases than did single-cell tumor emboli. If lymphocytes clump or aggregate around tumor cells, then these larger emboli would be more readily trapped in a capillary bed of a distant organ. An increase in the initial arrest of tumor emboli will result in a higher number of subsequent metastases (6, 11, 12). A possible relationship between circulating lymphocytes and tumor cells in vivo has been demonstrated in studies in which mice were given i.v. or s.c. injections of isogeneic tumor cells and 2 days later a profound lymphopenia occurred. However, no such lymphopenia occurred following tumor cell injection if the mice were immunized to the tumor (16). It is of interest to mention an incidental observation reported some time ago regarding the studies of cancer cells in blood of human patients with spontaneous neoplasia. In this report, several figures have been published demonstrating clumps of tumor cells isolated from the blood of tumor patients. Upon closer examination, lymphocytes were seen adhering to the tumor cells and their presence is noted in the legends of Figs. 1 and 2 (24).

Our experiments demonstrated that a low number of
normal or sensitized lymphocytes mixed with B16 melanoma cells could increase the incidence of pulmonary metastases. Once a critical dose is exceeded, immunized lymphocytes, while still clumping the tumor cells, were also cytotoxic. These findings closely support the hypothesis and experiments by Prehn (27–29) that weakly antigenic tumors or a weak incipient immune response may bring about cellular immune reactivity which is beneficial to tumor cell growth. As we described in our earlier studies (8), tumor cells which readily metastasize might do so because they possess characteristics allowing for their survival. It is possible that immune-mediated stimulation to metastasis as seen in our studies may aid in cancer growth and dissemination in vivo.

REFERENCES

Figs. 1 and 2. Clumping of B16 melanoma cells by syngeneic sensitized C57BL/6 lymphocytes (1000:1 lymphocytes to tumor cell) following 90 min incubation in vitro. Note clustering of tumor cells and adherence of lymphocytes to their surface. × 450.
Immune Stimulation-Inhibition of Experimental Cancer Metastasis

Isaiah J. Fidler

Cancer Res 1974;34:491-498.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/34/3/491

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.