Effect of Cyclophosphamide on Survival of Mice and Incidence of Metastatic Tumor following Intravenous and Intracardial Inoculation of Tumor Cells

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ABSTRACT

We studied the effect of cyclophosphamide on survival of mice and the incidence of tumor implants in various organs following both i.v. and intraarterial dissemination of tumor cells. Female C3H/HeN mice received cyclophosphamide (240 mg/kg) i.p. 4 days prior to inoculation of various doses of KHT tumor cells. Mice were followed to death, and the amount of tumor present was roughly quantified. Following i.v. inoculation of tumor cells, survival was decreased in cyclophosphamide-treated mice compared to control mice. However, survival was not affected by treatment with cyclophosphamide in mice receiving intracardial tumor cell injections. Pretreatment with cyclophosphamide caused a dramatic increase in the number of lung tumor implants following both routes of tumor cell administration. A similar tumor-promoting effect by cyclophosphamide could not be documented in the brain, heart, kidney, adrenal, or ovary. The study suggests that cyclophosphamide has a much greater effect on ultimate deposition and growth of tumor implants in the lungs than in other systemic organs or in the central nervous system.

INTRODUCTION

Because of its proximity to the external environment, the lung is an organ with well-developed mechanisms (both mechanical and immunological) for surveillance against invasion by a variety of foreign materials and pathogens. It is also the initial recipient organ of any substance or cell population which has been injected i.v. These 2 factors plus the anatomical accessibility of the lungs have meant that most experimental work elucidating inhibition of tumor growth on tumor implantation in the brain and other systemic organs. For this purpose, we chose cyclophosphamide, a drug which has been extensively studied. At certain dose levels, cyclophosphamide will augment pulmonary tumor growth after i.v. inoculation of tumor cells (9, 12, 13), as well as increase the number of metastatic tumor takes in the lungs after s.c. or i.m. injection of tumor cells (2, 8). Other studies, including adoptive transfer experiments and experiments using antibodies against NK cells, suggest that the effect of cyclophosphamide whereby it can promote tumor growth in the lungs is a result of NK cell suppression (13, 14, 25).

In order to design an experiment which would represent the reverse of our previous work, we wanted an agent which, as one of its actions, would suppress NK cell activity and explore its effect on the development of tumor implants in the brain and other systemic organs. For this purpose, we chose cyclophosphamide, a drug which has been extensively studied. At certain dose levels, cyclophosphamide will augment pulmonary tumor growth after i.v. inoculation of tumor cells (9, 12, 13), as well as increase the number of metastatic tumor takes in the lungs after s.c. or i.m. injection of tumor cells (2, 8). Other studies, including adoptive transfer experiments and experiments using antibodies against NK cells, suggest that the effect of cyclophosphamide whereby it can promote tumor growth in the lungs is a result of NK cell suppression (13, 14, 25).

Work reporting the effect of cyclophosphamide on metastases to sites other than the lung is very sparse. In the experiments reported here, we have looked at the effect of treatment with cyclophosphamide on the incidence of tumor implants in both the lungs and other organs. Our results show that, while cyclophosphamide had a dramatic effect on the yield of lung tumor nodules, it had little or no impact on tumor nodules forming in the brain, heart, ovary, kidney, or adrenals after i.v. or i.c. tumor cell inoculation.

MATERIALS AND METHODS

Mice. Female C3H/HeN mammary tumor virus-negative mice were obtained from Simonson Laboratories, Gilroy, CA. Mice used were between 6 and 10 weeks of age and were age matched within a single experiment.

Treatment with Cyclophosphamide. Contents of vials containing 20 mg of cyclophosphamide and 90 mg of sodium chloride (Mead Johnson Laboratories, Evansville, IL) were dissolved in 10 ml of sterile water. Treated mice received cyclophosphamide (240 mg/kg) by i.p. injection 4 days prior to i.v. or i.c. inoculation of tumor cells or harvesting spleen cells for in vitro cytotoxicity assays (13). Control mice received 0.2 ml of sterile water or 0.2 ml of 0.9% NaCl solution (saline) by i.p. injection.

Evaluation of Treatment with Cyclophosphamide on Body Weight in Tumor-bearing Mice. To determine the effect of cyclophosphamide on body weight, 30 control mice and 30 mice treated with cyclophos-
phamide were weighed. Four days after cyclophosphamide treatment, all animals were reweighed, and 17 control mice and 16 cyclophosphamide-treated mice were given injections of $1 \times 10^6$ tumor cells i.v. The remaining mice received $1 \times 10^4$ tumor cells i.c. All mice were weighed every 3 days until death.

**Evaluation of Cyclophosphamide Effect on the Weights of Specific Organs.** The effect of cyclophosphamide and tumor cell inoculation on the weight of certain organs was studied by injecting tumor cells 4 days after cyclophosphamide treatment and subsequently sacrificing animals at various intervals during the ensuing 48 hr. Two groups of 25 cyclophosphamide-treated and 25 control mice received either $1 \times 10^5$ tumor cells i.v. or i.c. It is known that there is no discernible tumor growth in any of the organs during the first 48 hr following cell inoculation (6). At 1, 4, 8, 24, and 48 hr after tumor cell inoculation, subgroups of 5 animals each were sacrificed with CO$_2$ and the brain, heart, lungs, ovaries, spleen, kidneys, and adrenals were harvested. The organs from each animal were fixed in fixative [37 to 40% formaldehyde (10 ml)-80% ethyl alcohol (90 ml)-glacial acetic acid (5 ml)] and weighed to the nearest 0.01 g.

Tumor. Cell lines of the KHT mouse sarcoma, a tumor that arose spontaneously in a C3H mouse, were grown in Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, NY) supplemented with 20% fetal calf serum, l-glutamine, sodium bicarbonate, and kanamycin (1 g/100 ml). Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO$_2$. All studies were done with cell populations in the exponential growth phase. Tumor cells were harvested by incubation for 15 min with 3 ml of 0.01% trypsin (Millipore Corp., Bedford, MA) and washed in Eagle's minimal essential medium with 20% fetal calf serum and then with Hank's balanced salt solution. They were resuspended in Hank's solution, and the viable cell count was adjusted to the desired concentration. Only cell suspensions with greater than 90% viability as determined by trypan blue exclusion were used. Mice received $1 \times 10^5$, $1 \times 10^4$, or $1 \times 10^3$ tumor cells in 0.2 ml by lateral tail vein injection or by a direct i.e. injection technique described previously (5).

**Procedure for Assay of Tumor Nodules after i.v. Inoculation of Tumor Cells.** In 3 of 4 experiments, cyclophosphamide-treated or control mice in groups of 7 to 10 received 1 $\times 10^5$ or 1 $\times 10^4$ tumor cells i.v. Animals were observed daily and followed to death or until 60 days after tumor cell inoculation, at which time they were sacrificed with CO$_2$. In the fourth experiment, 17 control and 16 cyclophosphamide-treated mice received $1 \times 10^5$ tumor cells i.v. Eighteen days later, all except 5 mice from each group were sacrificed with CO$_2$. The remaining 5 animals per group were followed to death as above. Animals from all experiments were autopsied, and the brain, heart, lungs, ovaries, kidneys, and adrenals were collected and fixed in formaldehyde-ethyl alcohol-glacial acetic acid fixative. To roughly quantify the number of tumor implants present in each organ, surface tumor nodules were counted using a dissecting microscope. Tumor growth was confluent when there were more than 100 metastatic tumor nodules per lung or 25 nodules per heart. Therefore, any lung or heart with this many or more tumor nodules was labeled too numerous to count. For statistical purposes, a value of 100 tumor nodules for lungs and 25 for hearts was used for organs labeled too numerous to count.

**Procedure for Assay of Tumor Nodules after i.c. Inoculation of Tumor Cells.** In 3 experiments, cyclophosphamide-treated or control mice in groups of 4 to 6 received 1 $\times 10^5$ tumor cells i.c. In the fourth experiment, 5 control mice and 5 cyclophosphamide-treated mice received $1 \times 10^5$ tumor cells i.c. All animals were observed daily until death. In a fifth experiment, 15 cyclophosphamide-treated and 14 control mice received $1 \times 10^5$ tumor cells i.c. By 12 days after tumor cell injection, 2 mice in each group were dead. Of the remaining mice, all but 5 from each group were sacrificed with CO$_2$. The remaining 5 mice per group were followed to death. Animals from all experiments were autopsied. Organs were harvested, and tumors were quantitated as outlined above. Mice with missed i.c. injections of tumor cells were readily identified by the presence of a large mediastinal tumor mass with little tumor elsewhere. Such mice were eliminated from all tabulations of results.

**In Vivo Assay for NK Cell-mediated Cytotoxicity.** To determine the sensitivity of the adherent KHT tumor line to killing by NK cells, we used the 24-hr in vitro cytotoxicity assay initially described by Shiku et al. (27) and modified by Hanna and Burton (12) and Hanna and Fidler (15). KHT tumor target cells prelabeled with $[^{3}H]$proline (I-cis-3-H, $[^{3}H]$proline; specific activity, 20, 40 Ci/mmol; New England Nuclear, Boston, MA) were dispersed into flat-bottomed wells ($10^5$ cells/well) of a Microtest culture plate (Falcon Plastics, Oxnard, CA) and allowed to adhere for 4 hr prior to testing. Single cell suspensions of effector cells were obtained from spleens of normal mice and mice given i.p. injections of cyclophosphamide 4 days previously. Effector cells were added ($10^5$ cells/well) to quadruplicate wells and incubated with the target cells for 24 hr in a humidified incubator with 5% CO$_2$ at 37°C. The wells were thoroughly washed with warm medium, and the remaining viable adherent cells were lysed with 3% sodium dodecyl sulfate. The contents of the wells were transferred to glass vials containing 10 ml of phase-combining scintillation fluid, and the amount of radioactivity was monitored in a Beckman scintillation counter. The percentage of killing was calculated by the formula

$$\% \text{ of cytotoxicity} = 1 - \frac{\text{cpm remaining in experimental wells}}{\text{cpm remaining in medium-control wells}}$$

**Statistics.** Statistical evaluations for significance were done by Student's t test.

**RESULTS**

**Effect of Treatment with Cyclophosphamide on Survival, Body Weight, and Selected Organ Weights.** A group of mice that received a single i.p. dose of cyclophosphamide (240 mg/kg) was followed for 60 days without mortality or obvious physical disability. Cyclophosphamide had no significant effect on body weight 4 days after a single i.p. dose of cyclophosphamide (240 mg/kg).

To study the effect of cyclophosphamide on the body weight of tumor-bearing mice, cyclophosphamide-treated and control animals received $1 \times 10^5$ tumor cells i.v. or $1 \times 10^4$ tumor cells i.c. and were weighed every 3 days until death. In this experiment, treatment with cyclophosphamide had no significant effect on the weight of mice receiving i.c. tumor cells; these mice died 14 days after tumor cell inoculation, 18 days after treatment with cyclophosphamide. Cyclophosphamide-treated mice receiving tumor cells i.v. had a significantly lower body weight beginning 15 days after tumor cell inoculations, 19 days after treatment with cyclophosphamide.

We also studied the effect of cyclophosphamide on the weights of specific organs over a short time period. Because weights of individual organs within each organ group did not change at the time periods sampled during the 48 hr after tumor cell injections, the weights of each group of organs for both control and cyclophosphamide-treated mice have been pooled. The only organs the weights of which were affected by cyclophosphamide treatment were the spleens and the ovaries. In cyclophosphamide-treated mice, there was a significant decrease in weight in both organs with the weight of the spleens falling 46% when compared to controls. Cyclophosphamide had no effect on the weight of the brain, lungs, kidneys, or adrenals.

**Effect of Treatment with Cyclophosphamide on Survival after i.v. Inoculation of Tumor Cells.** Although survival times varied between experiments, results were in the same direction...
for each dose of tumor cells in each experiment, so results have been pooled in Table 1. Treatment with cyclophosphamide significantly decreased survival of tumor-bearing mice. In the cyclophosphamide-treated groups, survival was shortened by an average of 5 days when compared to control mice (p < 0.005).

Effect of Treatment with Cyclophosphamide on the Incidence of Tumor Implants in the Lungs, Brain, and Other Systemic Organs following i.v. Tumor Cell Inoculation. There was considerable variation between experiments in the number of tumor implants that developed after i.v. tumor cell inoculation, but with both doses of tumor cells, and in animals sacrificed at Day 18 and those followed to death, results varied in the same direction in all experiments, and results have been pooled. Table 2 presents the pooled data and shows a marked increase in the number of tumor nodules in the lungs of cyclophosphamide-treated mice when compared to control mice (P < 0.005). For example, at a dose of 1 x 10^3 tumor cells injected i.v., normal animals developed a mean of 3.4 ± 1.0 lung tumor nodules compared to 51.7 ± 2.3 lung tumor nodules in cyclophosphamide-treated animals. As shown in Table 2, only a small number of tumor implants developed in the heart, ovary, kidneys, adrenals, or brain after i.v. inoculation of tumor cells. In these organs, the number of tumor implants was too small to allow meaningful statistical comparison between control and cyclophosphamide-treated mice. Tumor implants were not found in the livers or spleens in any of the mice, treated or not.

Effect of Treatment with Cyclophosphamide on Survival in Mice Receiving Tumor Cells by i.c. Inoculation. In order to bypass the trapping effect of the lung following i.v. inoculation of tumor cells and to deliver appreciable numbers of tumor cells to organs other than the lung, we used a left ventricular injection technique for inoculating tumor cells. A total of 4 experiments was done using a tumor cell dose of 1 x 10^4, and one experiment used 1 x 10^5 tumor cells. Because results from different experiments were similar, the results from the experiments using 1 x 10^4 tumor cells have been pooled. Unlike the effect of treatment with cyclophosphamide on survival in animals receiving tumor cells i.v., cyclophosphamide had no appreciable effect on survival of mice at either of the tumor cell doses injected i.c. (Table 3).

**Effect of Treatment with Cyclophosphamide on the Incidence of Tumor Implants in the Lungs, Brain, and Other Systemic Organs following i.v. Tumor Cell Inoculation.**

<table>
<thead>
<tr>
<th>Tumor cell dose</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Lung*</th>
<th>Heart</th>
<th>Ovaries</th>
<th>Kidneys</th>
<th>Adrenals</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10^4</td>
<td>None</td>
<td>24</td>
<td>34 ± 1.0</td>
<td>0.25 ± 0.17</td>
<td>0.17 ± 0.10</td>
<td>0</td>
<td>0</td>
<td>0.2 ± 0.13</td>
</tr>
<tr>
<td>1 x 10^4</td>
<td>Cyclophosphamide</td>
<td>24</td>
<td>51.7 ± 2.3</td>
<td>0.17 ± 0.13</td>
<td>0.13 ± 0.07</td>
<td>0.04 ± 0.04</td>
<td>0.4 ± 0.04</td>
<td>0.9 ± 0.22</td>
</tr>
<tr>
<td>1 x 10^5</td>
<td>None</td>
<td>18</td>
<td>44.6 ± 12.0</td>
<td>0.10 ± 0.08</td>
<td>0.11 ± 0.08</td>
<td>0</td>
<td>0</td>
<td>0.7 ± 0.28</td>
</tr>
<tr>
<td>1 x 10^5</td>
<td>Cyclophosphamide</td>
<td>17</td>
<td>100.0 ± 0</td>
<td>0.06 ± 0.06</td>
<td>0.12 ± 0.12</td>
<td>0.06 ± 0.06</td>
<td>1.1 ± 0.38</td>
<td></td>
</tr>
</tbody>
</table>

* At death, more than 100 tumor nodules per lung could not be counted accurately. Therefore, for any lung with more than 100 tumor nodules, a value of 100 was used for statistical analysis.

**DISCUSSION**

The fact that cyclophosphamide can increase the number of pulmonary nodules that develop after i.v. tumor cell administration has been well documented and extensively studied, and our results are consistent with these prior observations (1, 9, 12). The effect of cyclophosphamide on the number of tumor implants involving other organs has received far less investigational effort. Although we counted more pulmonary tumor nodules in mice after i.v. than i.c. tumor cell inoculation, we were able to document a relatively greater tumor-promoting effect of pretreatment with cyclophosphamide after i.c. tumor cell injections. Following i.c. administration of tumor cells, there was a dramatic 50- to 80-fold increase in the number of pulmonary nodules in cyclophosphamide-treated mice compared to control mice. However, by counting surface tumors in other organs, we were unable to document a similar tumor-promoting effect by cyclophosphamide.

**Effect of Treatment with Cyclophosphamide on Survival after i.v. KHT Tumor Cell Inoculation**

<table>
<thead>
<tr>
<th>Tumor cell dose</th>
<th>No. of mice</th>
<th>Survival (days)</th>
<th>No. of mice</th>
<th>Survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10^4</td>
<td>19</td>
<td>14.6 ± 0.43</td>
<td>18</td>
<td>14.6 ± 0.43</td>
</tr>
<tr>
<td>1 x 10^5</td>
<td>5</td>
<td>11.5 ± 0.29</td>
<td>4</td>
<td>11.5 ± 0.29</td>
</tr>
</tbody>
</table>

* Mean ± S.E.

Effect of Treatment with Cyclophosphamide on the Incidence of Tumor Implants following i.c. Tumor Cell Inoculation. As shown in Table 4, there was a 50- to 80-fold increase in the number of artificial tumor implants in the lungs of cyclophosphamide-treated mice following i.c. tumor cell inoculation. Treatment with cyclophosphamide had no significant effect on the number of tumor nodules developing in the heart, ovaries, kidneys, adrenals, or brain at either dose of tumor cells. Tumor implants to the liver, spleen, or peritoneum were not observed in any mice, treated or not.

**Assessment of NK Cell-mediated Cytotoxicity.** In a 24-hr in vitro cytotoxicity assay, KHT tumor target cells were killed by spleen cells from normal mice. However, cyclophosphamide given to mice at a dose of 240 mg/kg 4 days prior to harvesting spleen cells virtually eliminated NK cell activity against the KHT tumor cells (Table 5).

**Table 1**

<table>
<thead>
<tr>
<th>Tumor cell dose</th>
<th>No. of mice</th>
<th>Survival (days)</th>
<th>No. of mice</th>
<th>Survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10^4</td>
<td>13</td>
<td>28 ± 0.95</td>
<td>13</td>
<td>23 ± 0.00</td>
</tr>
<tr>
<td>1 x 10^5</td>
<td>19</td>
<td>24.5 ± 0.27</td>
<td>17</td>
<td>19.2 ± 0.63</td>
</tr>
</tbody>
</table>

* Mean ± S.E.  

**Table 2**

<table>
<thead>
<tr>
<th>Tumor cell dose</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Lung*</th>
<th>Heart</th>
<th>Ovaries</th>
<th>Kidneys</th>
<th>Adrenals</th>
<th>Brain</th>
</tr>
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<tbody>
<tr>
<td>1 x 10^4</td>
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<td>0</td>
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<tr>
<td>1 x 10^4</td>
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<td>0.13 ± 0.07</td>
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<td>None</td>
<td>18</td>
<td>44.6 ± 12.0</td>
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<td>0</td>
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<tr>
<td>1 x 10^5</td>
<td>Cyclophosphamide</td>
<td>17</td>
<td>100.0 ± 0</td>
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<td>1.1 ± 0.38</td>
<td></td>
</tr>
</tbody>
</table>

* At death, more than 100 tumor nodules per lung could not be counted accurately. Therefore, for any lung with more than 100 tumor nodules, a value of 100 was used for statistical analysis.

**Table 3**

<table>
<thead>
<tr>
<th>Tumor cell dose</th>
<th>No. of mice</th>
<th>Survival (days)</th>
<th>No. of mice</th>
<th>Survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10^4</td>
<td>19</td>
<td>14.6 ± 0.43</td>
<td>18</td>
<td>14.6 ± 0.43</td>
</tr>
<tr>
<td>1 x 10^5</td>
<td>5</td>
<td>11.5 ± 0.29</td>
<td>4</td>
<td>11.5 ± 0.29</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
Cyclophosphamide and Metastatic Tumor

Table 4
Effect of cyclophosphamide on the natural cytotoxicity of spleen cells against KHT tumor target cells in vitro

<table>
<thead>
<tr>
<th>Tumor cell dose</th>
<th>Drug treatment</th>
<th>No. of animals</th>
<th>Lung (%)</th>
<th>Heart (%)</th>
<th>Ovaries</th>
<th>Kidneys</th>
<th>Adrenals</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10⁴</td>
<td>None</td>
<td>26</td>
<td>1.3 ± 0.38</td>
<td>12 ± 1.65</td>
<td>1.1 ± 0.16</td>
<td>15.9 ± 3.88</td>
<td>1.64 ± 0.10</td>
<td>9.1 ± 1.1</td>
</tr>
<tr>
<td>1 x 10⁵</td>
<td>Cyclophosphamide</td>
<td>26</td>
<td>63.4 ± 6.56</td>
<td>13.8 ± 1.8</td>
<td>1.0 ± 0.18</td>
<td>22.9 ± 3.55</td>
<td>1.7 ± 0.10</td>
<td>7.6 ± 2.9</td>
</tr>
<tr>
<td>1 x 10⁶</td>
<td>None</td>
<td>5</td>
<td>1.2 ± 0.8</td>
<td>23.6 ± 0.68</td>
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<td>0</td>
<td>1.8 ± 0.2</td>
<td>15.6 ± 3.5</td>
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<tr>
<td>1 x 10⁷</td>
<td>Cyclophosphamide</td>
<td>4</td>
<td>100.0 ± 0</td>
<td>25 ± 0</td>
<td>2.3 ± 0.33</td>
<td>0</td>
<td>2.0 ± 0</td>
<td>16.5 ± 2.9</td>
</tr>
</tbody>
</table>

* At death, more than 100 tumor nodules per lung could not be counted accurately. Therefore, for any lung with more than 100 tumor nodules, a value of 100 was used for statistical analysis.

** At death, more than 25 tumor nodules per heart could not be counted accurately. Therefore, for any heart with more than 25 tumor nodules, a value of 100 was used for statistical analysis.

† Average ± S.E.

‡ Significantly different from controls (p < 0.05).

Our results suggest that there is an important difference in the way cyclophosphamide affects tumor growth in the lungs and in the other organs we studied. However, our results are at odds with those of Hanna and Fidler (13) who found that, following i.v. inoculation of B16 melanoma tumor cells, cyclophosphamide-treated mice had not only an increased number of nodules in the lungs but also developed nodules of melanoma in the liver, lymph nodes, kidneys, and peritoneum. Their control mice had tumor only in the lungs. Experience in our laboratory with the B16 melanoma in syngeneic mice suggests that this tumor readily and widely metastasizes when a large pulmonary tumor load is present. Also, following i.v. injection of a nonselected B16 tumor line, we have observed tumor implants in both the peritoneum and liver but never in the parenchyma of the CNS (tumor implants will very occasionally form in the leptomeninges). We question whether the increase in nonpulmonary tumors seen by Hanna and Fidler represents a cascade effect of tumor metastases and be merely a manifestation of a much larger pulmonary tumor burden in their cyclophosphamide-treated mice and not a direct effect of cyclophosphamide at the target organ level outside of the lung. Unfortunately, a direct comparison of their work and ours is impossible, because of the different metastatic behavior between the B16 melanoma and KHT tumor lines. The KHT tumor virtually never forms tumor implants in the liver, lymph nodes, peritoneum, or spleen following either i.v. or i.v. administration of tumor cells but readily implants in the neural parenchyma following i.c. inoculation of tumor cells.

Other of our data also suggest lack of a cyclophosphamide tumor-promoting effect on organs other than the lungs. In particular, our model has special utility for assessing the effect of systemically administered cyclophosphamide on the growth and development of tumor metastases in the brain. Work by others has demonstrated that significant amounts of cyclophosphamide and its metabolites cross the blood-brain barrier in normal brain (11, 23, 28) and that the quantity of drug is increased to values equivalent to those in serum in tumor tissue in the CNS (23).

After i.v. tumor cell inoculation of the KHT sarcoma, death is caused by progressive tumor growth in the lungs in both control and treated mice. Shortening of survival by pretreatment with cyclophosphamide is not surprising, since we documented a marked increase in pulmonary tumor burden in our treated animals. But following i.c. inoculation of tumor cells where mice die from progressive tumor growth in the brain rather than from pulmonary tumor growth, there was no difference in survival between cyclophosphamide-treated and control mice at any dose of tumor cells. Body weight loss was accelerated only in cyclophosphamide-treated mice receiving i.v. inoculation of tumor cells and not in those receiving i.c. inoculation of tumor cells. Also, the number of tumor colonies on the surface of the brain was the same in treated and control mice following i.c. inoculation of tumor cells at both doses of tumor cells studied. These observations argue against cyclophosphamide having a tumor-promoting effect within the CNS.

The mechanism whereby cyclophosphamide increases the incidence of pulmonary metastases is not entirely clear but most likely is caused by some alteration of the immune system and not from nonspecific generalized drug toxicity (8). The dose regimen used in our study did not cause obvious systemic toxicity; treated mice showed no change in whole-body weight when compared to controls, and the weights of most individual organs were not affected by cyclophosphamide. The possibility that the increase in pulmonary tumor nodules reflects nonspecific damage to the pulmonary vascular endothelium or parenchyma leading to increased arrest of tumor cells in the lungs is refuted by data from Hanna and Fidler (13) and Hanna and Burton (12). They saw no increase in the number of tumor cells that arrested in the lungs of cyclophosphamide-treated mice when compared to controls but were able to document prolonged survival of those tumor cells that did arrest.

Treatment with cyclophosphamide severely alters the immune system, and the relative effect of cyclophosphamide on various subcomponents of the immune system is complex. In our work, cyclophosphamide caused a 46% reduction in splenic weight by 4 days after treatment. This observation is consistent with earlier experimental work which additionally demonstrated almost total recovery of the splenocyte population in numbers and function by 10 to 14 days after cyclophosphamide treatment (19, 22). The results of our in vitro NK cell cytotoxicity assay demonstrate that the KHT tumor is sensitive to killing by NK cells and that in vitro NK cell cytotoxicity against KHT is virtually eliminated by pretreatment with cyclophosphamide. Of interest is a series of investigations by others, which suggest that activation of suppressor T-cells or other alteration of T-cell function by cyclophosphamide is not primarily responsible for its observed tumor-promoting effect (3, 8-10, 13, 21, 29) and also argue against a direct effect by cyclophosphamide on the unstimulated tissue.
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macrophage (1, 20, 25). However, Mantovani et al. (22), as well as the results from the present study, have demonstrated marked depression of NK cell activity in cyclophosphamide-treated mice, and Riccardi et al. (25) and Hanna and Fidler (14) demonstrated repair of this functional NK cell defect by adoptive transfer of splenocytes or pulmonary effector cells with high NK activity. Brooks et al. (2) studied the susceptibility of tumor cells from metastases at different sites to NK cell-mediated cytotoxicity, and they found that treatment with cyclophosphamide depleted the NK cell population in the lung but had little effect in other organs. Their work suggests that, in the lungs, pretreatment with cyclophosphamide may reduce the cytotoxic effect of NK cells, thus leading to an increased survival of tumor emboli and ultimate growth of more pulmonary tumors.

There are other immunomodulating agents which, rather than depressing NK cell activity, augment it. There is increased NK cell activity in mice treated with C. parvum or chronically infected with Toxoplasma gondii (16, 18, 26). Pretreatment with both agents inhibits the development of KHT tumor implants at multiple systemic sites, and Toxoplasma infection will also inhibit metastatic brain tumor (4). Our recent work in which we demonstrated that both C. parvum and chronic Toxoplasma infection reduced the number of circulating tumor cell emboli to multiple organs suggested that one mechanism whereby these immunomodulating agents protected an animal from developing metastatic tumor implants was by activation of NK cells (6). Herberman and Holdin (17) and Oehler and Herberman (24) have found that NK cell activation by these agents probably is indirect and involves interferon production by macrophages and other cells which, in turn, augment NK cell activity. It is still not known whether NK cells protect only against circulating tumor cells or whether, or another population of cells, can also be induced to kill tumor cells at a tissue level within the parenchyma of a particular organ.

In conclusion, the results of this study reveal that, in our system, the tumor-promoting effect of cyclophosphamide is quite specific for the lung, and the agent does not promote the development and growth of tumor implants in the brain, heart, ovary, kidney, or adrenal. We submit that any investigation of an agent being used to effect metastatic tumor implantation and growth (either to increase or decrease the amount) must include consideration of the brain and other systemic organs in addition to the lungs.

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Effect of Cyclophosphamide on Survival of Mice and Incidence of Metastatic Tumor following Intravenous and Intracardial Inoculation of Tumor Cells

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