Effect of Pertussis Antigen and Cyclophosphamid on Myeloma Tumor

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SUMMARY

Cyclophosphamide treatment and a combination of pertussis vaccine and cyclophosphamide treatment of transplanted mouse myeloma have been compared. Both treatments reduced mortality from the transplanted cancer. The combination of pertussis vaccine plus cyclophosphamide, although permitting a higher early mortality, resulted in a lower overall mortality. It also markedly reduced chances of a late running and wasting syndrome accompanying late mortality observed in mice treated with cyclophosphamide alone. Neither treatment influenced cellular or humoral immune reactions when they were evaluated 200 to 250 days after treatment.

INTRODUCTION

Host defenses against cancer have been studied extensively over the last decade. Some investigators (7, 9, 10, 39, 41, 42) have suggested that the host defense against malignant cells involves stimulation of the RES, especially the macrophages. For example, the influence of stimulation of the RES with Bacillus Calmette-Guérin on the outcome of transplanted cancer has been studied (8, 19, 20, 40). Encouraging preliminary results have been presented both in experimental animals and in man with a combination of immunotherapy and chemotherapy for the treatment of leukemia (2, 17, 28–30).

Many questions have been raised by these probes. Two of these seem particularly pressing: (a) can immunotherapy be beneficial in treatment of malignant diseases other than the leukemias and (b) can nonspecific immunotherapy be used to reduce adverse effects of chemotherapeutic agents on a host bearing a malignant tumor? These questions stimulated us to compare the treatment of a transplantable myeloma with large doses of an alkylating agent alone or in combination with prior stimulation with pertussis antigen.

Arnold et al. (4), Brock (11), Brock and Hohorst (12, 13), and Hohorst et al. (22) showed that cyclophosphamide has a good therapeutic index and that in sublethal doses its damage to normal tissues and its toxic effect on hematopoietic tissues are readily reversible. It is known that cyclophosphamide affects all cells of the lymphoid series (48) but is more toxic for proliferating cells and less toxic for certain stem cells and mature cells (6). Further, cyclophosphamide does not seem to affect adversely the function of macrophages (27).

Pertussis antigen (16, 24, 36–38), given i.v. to mice, mobilizes leukocytes from the lymphohemopoietic tissues to the peripheral blood and stimulates production of lymphoid cells. For a period after injection of pertussis antigen, the bone marrow and the spleen show a predominance of mature cells and all the lymphohemopoietic tissues are depleted of small lymphocytes. Pertussis antigen appears to provide the following influences which might be usefully combined with destruction of tumor cells produced by cyclophosphamide. It stimulates the RES (7, 9, 10, 39, 41, 42) and produces in lymphohemopoietic tissues a predominance of cells more resistant to injury by cyclophosphamide (6, 24); finally, it can stimulate specific immune responses (21, 26, 31, 43).

MATERIALS AND METHODS

Inbred BALB/c female mice were obtained from our own mouse colony, the stocks of which have been described previously (47). The mice were kept in air-conditioned animal quarters and nursed by their mothers until they were 4 weeks old, after which they were weaned and fed Purina fox chow and water ad libitum.

Groups of 50 to 104 animals were used to study the effect of cyclophosphamide and the effect of pertussis antigen plus cyclophosphamide on mice bearing a transplantable MT (Table 1).

An ADJ-PC5 transplantable MT (PC5 MT), capable of producing IgG2a, was obtained from Dr. M. Potter (National Cancer Institute Laboratory of Biology, Bethesda, Md.). Pertussis vaccine was obtained from Eli Lilly and Co. (Indianapolis, Ind.), and cyclophosphamide (Cytoxan) was obtained from Mead Johnson and Co. (Evansville, Ind.).

A large group of mice was given PC5 MT transplants approximately 2 x 2 mm in diameter s.c. in the right inguinal region. Cyclophosphamide was given i.p. in a total dose of 10 mg/mouse (~500 mg/kg) in divided doses on 4 successive days. On the 1st and 2nd day, each mouse received 3 mg/day; on the
Table 1  
Groups of mice used in study of influence of pertussis antigen and cyclophosphamide treatment of mice myeloma and early and late mortality

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Mean age at start of treatment (days)</th>
<th>Early mortalities (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Late mortalities (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC5 MT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50</td>
<td>62</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>PC5 MT + P</td>
<td>51</td>
<td>63</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>PC5 MT + C</td>
<td>104</td>
<td>66</td>
<td>13</td>
<td>53</td>
</tr>
<tr>
<td>PC5 MT + P + C</td>
<td>93</td>
<td>68</td>
<td>28</td>
<td>16</td>
</tr>
</tbody>
</table>

<sup>a</sup> From the day of transplantation to 20 days after treatment started.  
<sup>b</sup> Twenty-one days after treatment started and over.  
<sup>c</sup> PC5 MT, ADJ-PC5 myeloma tumor; P, pertussis antigen; C, cyclophosphamide.  

3rd and 4th day, each received 2 mg/day. Pertussis vaccine was given in an i.v. dose of 0.4 ml/mouse in a lateral tail vein.  

Mice transplanted with PC5 MT were divided into 4 groups (Table 1). One group was not treated, one group was treated only with pertussis antigen, another group was treated only with cyclophosphamide, and the last group was treated first with pertussis antigen and, 3 days later, with cyclophosphamide. We further divided the mice transplanted with PC5 MT into 4 subgroups selected on the basis of mean tumor diameter at the time cyclophosphamide was started (0.5 to 1.0 cm, 1.05 to 1.50 cm, 1.55 to 2.0 cm, and 2.05 cm and over).  

A group of normal mice and groups of mice given PC5 MT transplants previously and treated with cyclophosphamide or pertussis antigen plus cyclophosphamide were compared for cellular and humoral immunity by evaluation of allogeneic skin graft rejection and agglutinin production to killed Brucella organisms.  

RESULTS  

Tumor Growth. The effects of cyclophosphamide and pertussis antigen plus cyclophosphamide on the PC5 MT growth are summarized in Chart 1. In normal mice, 5 to 7 days after PC5 MT transplantation the growth of tumor is almost linear. Mice treated with pertussis antigen alone do not show significant deviation of their tumor growth from that observed in nontreated mice. In both of these groups, all mice died between 17 and 23 days after tumor transplantation. Mice transplanted with PC5 MT and treated first with pertussis antigen and then with cyclophosphamide or with cyclophosphamide alone show a steady decrease of the mean diameter of their tumor, which finally disappeared. The tumor decreased and disappeared more slowly in mice treated first with pertussis antigen and then with cyclophosphamide. This difference was most obvious in pertussis antigen-treated mice bearing a tumor of 1.55 to 2.00 cm at the time cyclophosphamide treatment was initiated.  

Body Weight. The body weight of normal mice at different ages and the body weight of mice transplanted with PC5 MT and treated with cyclophosphamide or with pertussis antigen plus cyclophosphamide are summarized in Chart 2. The changes of the body weight of mice treated at an early stage of tumor growth are not significantly different from changes observed in mice treated at an advanced stage of tumor growth. The body weight in mice transplanted with PC5 MT and treated either with cyclophosphamide alone or with pertussis vaccine plus cyclophosphamide shows a significant drop during the first 15 days after treatment. Mice treated with cyclophosphamide alone show a second drop of body weight between Days 60 and 130 after the cyclophosphamide treatment. Mice surviving this critical period show a steady increase of body weight. Both treated groups given PC5 MT transplants show a body weight significantly lower than that of normal mice.
Pertussis Antigen, Cyclophosphamide, and Malignancy

Mortality. The total early and late mortality observed in all treated experimental mice is shown in Table 1. The mortality in untreated mice and in mice treated only with pertussis vaccine after PC5 MT transplantation is 100%. These mice died between 17 and 23 days after tumor transplantation. The mortality in mice given PC5 MT transplants and treated with pertussis antigen plus cyclophosphamide or with cyclophosphamide alone is shown in Charts 3 and 4.

Mice bearing a tumor with a mean diameter of 0.5 to 1.5 cm and treated only with cyclophosphamide after PC5 MT transplantation show significantly higher mortality than that observed in the group treated first with pertussis antigen and then with cyclophosphamide ($X^2 = 53.8, p > 0.01$). In mice with a tumor of 0.5 to 1.0 cm at time of treatment, the total mortality in the group treated with cyclophosphamide alone is 60%; the total mortality of the group treated with pertussis antigen plus cyclophosphamide is only 25%. Similarly, in mice with tumors of 1.05 to 1.50 cm diameter, the total mortality rate in the pertussis antigen plus cyclophosphamide group is 40%, whereas 74% of those treated only with cyclophosphamide died. The mortality of mice bearing a tumor of 1.55 to 2.05 cm mean diameter and greater is almost the same as the mortality in mice treated either with pertussis antigen plus cyclophosphamide or with cyclophosphamide alone.

Of the total of 99 mice given PC5 MT transplants and treated only with cyclophosphamide, 65 (65.7%) died. Of 83 mice given PC5 MT transplants and treated with pertussis antigen plus cyclophosphamide, 36 died (43.4%) ($X^2 = 28.0, p < 0.05$). The differences between the two forms of treatment were significant if treatment was given when the tumors were small.

The critical period for survival after administration of cyclophosphamide is completely different for mice given PC5 MT transplants and treated with pertussis antigen plus cyclophosphamide and for mice treated only with cyclophosphamide (Table 1, Chart 4). Of these two groups, mice treated first with pertussis antigen and then with cyclophosphamide show the highest mortality (23 deaths) during the first 20 days of treatment, while mice treated only with cyclophosphamide show the highest mortality (49 deaths) between Days 60 and 170 after the administration of the cyclophosphamide. There seemed to be a direct relation between early mortality and the size of the tumor at the time the treatment was started.

The mice that died soon after treatment showed loss of weight in spite of developing generalized edema and hunched posture, ruffled fur, rapid respiration, and shivering. Their survival could be facilitated by special care, i.e., a clean and warm environment, moistened food in the cage, and long tubing on water bottles. Mice which died between Days 60 and 170 after administration of cyclophosphamide began to lose weight after having done quite well in the posttreatment period. They then lost almost 0.5 g every 2nd day and died within 17 to 22 days with a body weight of 11 to 12 g. Except for loss of weight, they did not appear to be ill, and in this way they seemed quite different from the mice which died early.

Histology. The histology of the tissues of mice dying long after treatment showed involuted lymph nodes, thymus, and...
spleen, degenerative changes in the wall of the gut, and scattered focal distribution of tumor cells in liver, spleen, lungs, and lymphoid tissues. Two mice treated with pertussis antigen and cyclophosphamide dying long after treatment had generalized lymphoma.

The histology of the tissues of mice dying during the early stage of treatment showed congestion of all organs, metastasis of the tumor cells, and pulmonary edema.

**Cellular and Humoral Immunity.** In an effort to determine why mice given transplants of PC5 MT and treated only with cyclophosphamide showed a very high late mortality, we studied the cellular and humoral immunity in survivors of both treated groups.

The time of allogeneic skin graft rejection of experimental animals and normal BALB/c female mice is compared in Table 2. No difference was observed in time of allograft rejection between normal mice and mice given PC5 MT transplants previously and treated either with pertussis antigen plus cyclophosphamide or with cyclophosphamide alone. One mouse out of 6 treated after PC5 MT transplantation with cyclophosphamide alone died 22 days after allogeneic skin graft with an intact transplant. This mouse was already wasted (body weight, 16 g) at the time of skin transplantation.

The humoral immunity of experimental and normal BALB/c female mice of the same age was tested with killed *Brucella* organisms. No significant difference was observed in antibody production against *Brucella* antigen between normal untreated mice; normal mice previously treated with pertussis vaccine, pertussis vaccine plus cyclophosphamide, or cyclophosphamide only (control groups); and mice previously given PC5 MT transplants and then treated with cyclophosphamide or with pertussis vaccine plus cyclophosphamide. In all instances, the prior treatment had been given more than 200 days before antigen stimulation.

**DISCUSSION**

We have shown that cyclophosphamide significantly suppresses mortality from transplanted myeloma in BALB/c mice. Even when cyclophosphamide therapy is begun when the tumors are large, the tumors rapidly melt away and disappear. Pertussis antigen treatment does not appreciably alter the influence of cyclophosphamide treatment on reduction of transplantable tumor size, although, when tumors are larger, pertussis antigen therapy seems to slow the reduction in size of the tumor mass to some extent. With the large dose of cyclophosphamide used in this study, early death of mice seemed more frequent when treatment was given to animals bearing small tumors. When treatment is given to animals bearing larger but still treatable tumors, early mortality is low, and death occurs primarily from a runting syndrome beginning 60 to 170 days after treatment. When cyclophosphamide is given at a time when tumors are very large and extensive, metastasis has already occurred, and early mortality may be high and, as in untreated animals, is associated with the widely disseminated cancer. The early deaths in mice bearing small tumors are especially confusing, since control mice given cyclophosphamide alone do not show this early death.

When the transplanted PC5 MT is relatively small, a combination of prior treatment with pertussis antigen and cyclophosphamide results in a significantly higher percentage of cures than does cyclophosphamide treatment alone. The rather striking synergistic influence of combined pertussis antigen and cyclophosphamide therapy was not observed in mice treated when tumor was very large or had disseminated widely.

Since pertussis antigen has many different influences, it is difficult to be certain which of its actions abets recovery from this transplanted cancer. Of interest in this regard is our finding that the death of animals with a transplanted cancer after a combination of pertussis antigen and cyclophosphamide therapy occurs early. By contrast, when such animals die following cyclophosphamide treatment alone, they tend to die later (60 to 170 days after treatment). Although the basis for the early and late deaths have not yet been thoroughly analyzed, the late deaths do not always seem to be attributable to dissemination of the tumor. Early deaths are associated with an acute wasting syndrome, which could be a function of toxicity derived from the influences of pertussis vaccine together with the effects of cyclophosphamide and the toxicity derived from the destruction of tumor cells. It is known, for example, that treatment with pertussis antigen markedly enhances susceptibility to histamine and anaphylactic shock (44—46). The edema of the pertussis antigen-treated mice dying early could be a hint that there is increased susceptibility to factors producing vascular alterations. Once the mice treated with pertussis antigen and cyclophosphamide after PC5 MT transplantation survive beyond 60 days, very few die and permanent cures seem to be the consequence.

It is quite different with tumor-bearing mice treated with cyclophosphamide alone. Except in the groups having the smallest or largest tumors at the time of treatment, early deaths were infrequent, and most animals dying succumbed with a runting and wasting syndrome between 60 and 170 days after treatment. Although tumor foci could be found in most animals autopsied, only in a very few did death seem attributable entirely to the tumor. It seems to us that either a reaction against the tumor or, more likely, an alteration of the host-parasite relation to the agent producing the cancer or to other infection might account for the wasting syndrome of these mice. Similar forms of late death with wasting and runting have been observed in neonatally thymectomized

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**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Days after C&lt;sub&gt;0&lt;/sub&gt; started</th>
<th>Rejection time (days)</th>
</tr>
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<tbody>
<tr>
<td>Normal (age, 250 days)</td>
<td>8</td>
<td></td>
<td>14.0</td>
</tr>
<tr>
<td>PCS5 MT + C (age, 200 days)</td>
<td>6</td>
<td>115</td>
<td>14.0</td>
</tr>
<tr>
<td>PCS5 MT + P + C (age, 218 days)</td>
<td>6</td>
<td>128</td>
<td>11.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> PCS5 MT, ADJ-PC5 myeloma tumor; C, cyclophosphamide; P, pertussis antigen.

<sup>b</sup> One graft not rejected and not included in the calculation of mean rejection time.
mice, irradiated thymectomized mice, or mice or rats experiencing chronic graft-versus-host reaction (1, 3, 5, 14, 15, 18, 23, 25, 32–35).

Further studies are needed to determine, by carefully timed autopsies, whether pertussis antigen treatment facilitates the ultimate elimination of minimal residual tumor, as has been proposed for other immunotherapeutic approaches in animals and man.

Whatever the basis of its influence, pertussis antigen therapy is worth further study as an adjunct to experimental tumor therapy and, possibly, as an adjunct to treatment of certain cancers in man.

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