Combined Cyclophosphamide Chemotherapy and Maltose Tetrapalmitate Immunotherapy in the Treatment of Transplanted Bladder and Prostate Carcinoma of the Rat

El Housseiny I. Ibrahiem, Vijai N. Nigam, Carlos A. Brailovsky, Brian D. Backman, and Mostafa M. Elhilali

Département d’Urologie [E. H. I. I., B. D. B., M. M. E.] et Département d’Anatomie et de Biologie Cellulaire [V. N. N., C. A. B.], Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Quebec, Canada J1H 5N4

ABSTRACT

Chemoimmunotherapy in two animal models for urological cancers was studied. The models were Dunning R3327A prostatic carcinoma transplanted s.c. in Fischer × Copenhagen F, hybrids and a well-differentiated bladder carcinoma transplanted orthotopically in the bladder submucosa of female Fischer rats. Cyclophosphamide, cis-platinum, and Adriamycin were initially used as anticancer chemotherapeutic agents, and the most effective ones were used in combination with maltose tetrapalmitate (MTP), which was used as an immunopotentiator. In the case of prostatic carcinoma, cyclophosphamide was the most effective among the anticancer agents in controlling tumor growth after inoculation of either 10⁶ or 10⁷ tumor cells. Combination of cyclophosphamide with i.p. MTP delayed tumor take and controlled tumor size more effectively than did either of the treatments given alone. Similar results were obtained in the case of bladder tumor. A combination of cis-platinum with MTP significantly controlled bladder tumor size, and a combination of cyclophosphamide with MTP cured 75% of the rats. The remaining 25% of this group had a small tumor that did not increase in size during the subsequent 2 weeks of observation without treatment. The incidence of metastasis of bladder tumor to lymph nodes and lung was reduced by MTP and cis-platinum and eliminated by cyclophosphamide alone and in combination with MTP. Nonspecific immunity as measured by phytohemagglutinin stimulation of spleen lymphocytes and antitumor immunity as measured by cytotoxicity and macrophage migration inhibition assays were highest in rats subjected to cyclophosphamide and MTP combined therapies.

INTRODUCTION

Combination of chemotherapy with agents that augment host antitumor immunity was soon brought into practice in the treatment of human neoplastic disease once it was apparent that the use of immunotherapy alone failed to live up to expectations. It was felt that a chemotherapeutic anticancer drug would: (a) reduce tumor load to a level whereby existing host antitumor immunity can eliminate residual tumor cells (28) or at least (b) slow tumor growth long enough to allow development of a potent antitumor response (7); (c) make tumor cells more susceptible to immune lysis by rendering them more immunogenic (3); and (d) potentiate cytotoxic immunity by elimination of immune suppressor mechanisms that interfere with the development of an effective antitumor immune response (15). Nonspecific immunotherapy would then act synergistically and enhance the developing antitumor immunity.

Progression of tumor growth has generally been attributed to either the appearance of suppressor elements that interfere with an effective antitumor response or an inefficient in vivo sensitization. These suppressor elements include blocking antibodies, free antigen shed by the tumor cells, or antigen-antibody complexes (2, 18). Such suppressor elements are well known to exist in the serum of tumor-bearing hosts and are known to be controllable by a decrease in tumor volume.

Animal experiments that followed clinical trials supported the hypothesis that combinations of chemotherapeutic agents with an immune stimulant such as Bacillus Calmette-Guérin, Corynebacterium parvum, or levamisole are superior than either of these treatments alone (7, 9). However, the outcome of the human experience has been less rewarding (16, 17, 21, 22).

The present study was undertaken to try out a new immunoadjuvant, MTP, in combination with chemotherapy. Previously, this agent had been found superior to several of the conventional immunoadjuvants with regard to delay in tumor appearance after tumor inoculation and tumor cell growth (12). It also prevented tumor recurrence when given to animals after tumor surgery (12). MTP has been found to be essentially nontoxic and nonimmunogenic (25).

In this work on chemoimmunotherapy, we used 2 urological tumor models to test the efficacy of MTP to potentiate the antitumor effects of 3 chemotherapeutic agents. The models used were: (a) a Dunning R3327A prostatic carcinoma, which was transplanted s.c., and (b) a well-differentiated N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide-induced transitional cell carcinoma of the bladder (BT4) transplanted orthotopically in the bladder submucosa. The results indicate that both of these tumors were more responsive to a combination of cyclophosphamide and MTP therapy than when either of these was instituted alone. Results were especially encouraging in the bladder tumor model in which chemoimmunotherapy could render a majority of animals tumor free.

MATERIALS AND METHODS

Rats. Inbred male and female Copenhagen rats were initially obtained from the National Cancer Institute, Bethesda, MD. Subsequently, they were reared in our animal colonies. The source of inbred Fischer 344/
CRBL rats was La Ferme et Laboratoire canadien d’élevage Ltée, St-
Constant, Québec, Canada. These were also bred and raised in our
facilities. F1 hybrid Copenhagen × Fischer rats were raised by cross-
breeding inbred male Copenhagen rats with inbred female Fischer 344/
CRBL rats. The animals were fed Purina chow and water ad libitum.

Chemicals. Cyclophosphamide (Bristol Laboratories of Canada, Can-
diac, Quebec, Canada), cis-platinum (Bristol Laboratories of Canada,
Candiac, Quebec, Canada), and Adriamycin (Adria Laboratories, Wil-
ington, DE) were purchased from our hospital pharmacy. MTP was
prepared as described previously (25).

Tumors and Their Transplantations. Dunning R3327 A prostatic ad-
carcinoma was kindly supplied by Dr. S. Coffey, Johns Hopkins Hospit-
al, Baltimore, MD, as a s.c. implant in male Copenhagen rats. It
was maintained by s.c. transplantation in the left flank of male Copenhagen
× Fischer F1 hybrid rats. The tumor cell suspension was prepared
in PBS by chopping solid tumor and obtaining single cell suspension.
The viability of tumor cells was tested by trypan blue exclusion.

The primary bladder tumor was derived from one of the female Fischer
rats fed N-(4-(5-nitro-2-furyl)-2-thiazolyl)formamide for 14 months. His-
tologically, the tumor was a transitional cell carcinoma. The tumor
was maintained by the orthotopic transplantation of viable tumor cells into
the bladder submucosa of female Fischer rats (20). The present experi-
ments were done when the tumor was in its fourth orthotopic passage.
In this work, it is abbreviated as BT4.

Tumor Size. Tumor size was determined for s.c. tumor by using a
caliper to measure the length and width of the tumor and was calculated
according to the formula

\[ \text{Tumor size} = L \times W^2 \times 0.4 \]

where \( L \) is the length and \( W \) is the width of the tumor in cm (1). Bladder
tumor size was determined by weighing the bladder. It has been reported
(29) that weight of bladder tumor is equivalent to the weight of the whole
Tumor-bearing bladder, because of the smallness of rat bladder weight
(50 to 70 mg).

Chemotherapy and Chemoimmunotherapy of Experiments with
Dunning Prostatic Tumor. Sixty male Copenhagen × Fischer hybrid rats
weighing 120 to 150 g were divided into 3 equal groups (20 rats each)
which received 104, 105, and 106 Dunning tumor cells s.c., respectively.
Each group was subdivided into a control group and 3 treatment groups
(5 rats each) which received either Adriamycin, cis-platinum, or cyclo-
phosphamide i.p. The doses and schedules used were: Adriamycin, 2.5
mg/kg/week for 4 weeks; cis-platinum, 10 mg/kg/week for 4 weeks;
and cyclophosphamide, 60 mg/kg/week for 4 weeks. The treatments
were given once weekly, after dissolution or suspension in PBS (0.2 ml).
The doses were selected from literature survey and from our mortality
observations at higher doses (19). The control animals received 0.2 ml
of PBS. The animals were examined daily for tumor development and
measurement of tumor size. The above study was carried out to choose the most effective anticancer agents among the 3 and to identify the smallest
tumor dose that was not completely eliminated by the anticancer drug.

For chemoimmunotherapy of prostatic tumor, the following experiment
was done. Sixty male Copenhagen × Fischer F1 hybrid rats (120 to 150
\( g \)) received 5 \( \times 10^4 \) Dunning tumors s.c. in the left flank. They were then
divided into 6 groups of 10 rats each. The first group received no
therapy. The remaining 5 groups received the following treatments:
Group 2, cyclophosphamide (60 mg/kg/week); Group 3, MTP (10 \( \mu g \) i.p./
day/rat); Group 4, MTP (20 \( \mu g \) i.p./day/rat); Group 5, cyclophosphamide
(as above) plus 10 \( \mu g \) of MTP i.p./day/rat; and Group 6, cyclophos-
phamide (as above) plus 20 \( \mu g \) of MTP i.p./day/rat. This i.p. dose of 10 to
20 \( \mu g \) of MTP was found to be optimal previously (25).

Initially, the tumor size was measured daily in order to determine the
time required for the tumor size to reach 1 cm; it was then measured
weekly to study the effects of treatment on tumor growth. Survival
times were determined; and autopsies were performed to study the effect
of treatment on tumor metastasis.

Chemotherapy and Chemoimmunotherapy Experiments with BT4.
One hundred female Fischer rats were each given injections of 25 \( \times 10^4 \)
BT4 cells in 0.1 ml of PBS into the submucosal layer of the bladder. Two
weeks later, the bladders of these rats were examined by transillumina-
tion to detect very early tumor vascular changes that identify tumors as
small as 1 mm in diameter. All of the animals exhibited presence of a
growing tumor in the experiment. The animals were then divided ran-
domly into 10 groups of 10 rats each. These groups were placed in the
following therapeutic regimens: Group 1 (control), no treatment; Group
2, MTP, 20 \( \mu g \)/rat/day p.o.; Group 3, cyclophosphamide, 30 mg/kg/rat;
Group 4, cyclophosphamide, 60 mg/kg; Group 5, cyclophosphamide, 30
mg/kg, plus MTP, 20 \( \mu g \)/rat/day p.o.; Group 6, cyclophosphamide, 60
mg/kg, plus MTP, 20 \( \mu g \)/rat/day p.o.; Group 7, cis-platinum, 5 mg/kg;
Group 8, cis-platinum, 10 mg/kg; Group 9, cis-platinum, 5 mg/kg, plus
MTP, 20 \( \mu g \)/rat/day p.o.; and Group 10, cis-platinum, 10 mg/kg, plus
MTP, 20 \( \mu g \)/rat/day p.o.

The chemotherapy was given once weekly for 4 weeks, and MTP was
given continuously in drinking water for 4 weeks. On the average, Fischer
rats (120 to 150 g), with or without tumors, drink 20 ml of water, as
observed by us in other experiments. Twenty g of MTP were contained
in 20 ml of drinking water. After 4 weeks of treatment, the animals were
left untreated for another 2 weeks. The animals were then subjected to
the examination of the bladder by transillumination to observe for vas-
cular changes, and rats were later sacrificed. The bladder was removed,
weighed, and fixed in formalin for later pathological examination. The
spleens of the animals were removed, and the spleen suspensions were
prepared for estimating nonspecific and antitumor specific immunities.

Immunological Studies. Blastogenic response of spleen lymphocytes
with phytohemagglutinin (Difco Laboratories, Detroit, MI) was carried out
as described by us previously (25). This test measures the afferent
lymphoproliferative limb of immune reactivity. For measurement of cell-
mediated antitumor immunity, a macrophage migration inhibition assay,
as described by David et al. (10), was used. The test antigen was
prepared as described by Svaboda et al. (30). In addition, lymphocyto-
toxicity of spleen cells against BT4 targets was estimated by the 31Cr
release assay as described by Brunner et al. (5). These 2 tests measure
2 different components of the efferent limb of the immune system.

Statistical Methods. The significance of the activity of the different
treatments on tumor development and on survival time was determined
with the single way analysis of variance test and Student’s t test.

RESULTS

Chemotherapy of Prostatic Carcinoma. Table 1 shows the
effect of treatment with cyclophosphamide, cis-platinum, and
Adriamycin on the time required for tumor appearance after
R3327 A tumor inoculation s.c. in 3 doses (10⁴, 10⁵, and 10⁶
cells) and the survival times of these animals. Chart 1 shows the
rate of tumor growth in the same experiment. As expected, the
lower tumor dose lengthened the time required for tumor ap-
pearance in both control and treated animals. Unexpectedly, the
tumor cell dose did not affect significantly the survival times of
these animals. The differences in tumor appearance time be-
tween untreated and Adriamycin- and cis-platinum-treated
animals were not significant. However, cyclophosphamide treat-
ment lengthened the tumor appearance time significantly as
compared to controls at a 10⁴ tumor cell dose, and tumors failed
to appear on cyclophosphamide treatment when 10⁵ tumor cells
were used for inoculation. There was no significant difference in
the survival times of animals that were left untreated and those
that were treated with Adriamycin or cis-platinum. On the other
hand, cyclophosphamide-treated animals had significant length-
ening in the survival time when compared to controls at the 10⁵
and 10⁶ tumor cell dose levels. Animals receiving 10⁴ tumor cells
Table 1

Effect of chemotherapy on the day of tumor appearance and on survival times of animals inoculated with various doses of prostatic carcinoma

For details, see "Materials and Methods."

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Day of tumor appearance (days)</th>
<th>Survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.68 ± 4.06b 28.09 ± 5.93</td>
<td>7.0 ± 0.0 26.4 ± 3.58</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>18.25 ± 2.19 30.80 ± 2.95</td>
<td>9.4 ± 1.9 34.2 ± 2.86</td>
</tr>
<tr>
<td>cis-Platinum</td>
<td>18.25 ± 3.4 31.0 ± 7.57</td>
<td>11.8 ± 1.34 33.6 ± 1.95</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>No tumors 54.40 ± 9.04b</td>
<td>14.0 ± 2.45 45.5 ± 4.72</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

a These rats died from infection without the presence of visible tumor or tumor metastasis.

Chart 1. Effect of chemotherapy on R3327 A tumor size in animals inoculated s.c. with $10^4$ (a), $10^5$ (b), and $10^6$ (c) R3327 A tumor cells, •, untreated controls; A, treated with Adriamycin; A, treated with cis-platinum; O, treated with cyclophosphamide (Cy). For details, see "Materials and Methods." Significant difference (Chart 2a): cyclophosphamide versus controls, p < 0.05.

Chart 2. Effect of cyclophosphamide and MTP chemoimmunotherapy on R3327 A tumor size (a) and percentage of tumor takes (b) as a function of time. Numbers in a, number of animals with tumor/total number of animals. •, untreated controls; A, treated with $10^6$ MTP; A, treated with $20 \mu$g of MTP; O, treated with cyclophosphamide; •, treated with cyclophosphamide plus MTP (10 $\mu$g); •, treated with cyclophosphamide plus MTP (20 $\mu$g). For details, see "Materials and Methods." Significant differences: Chart 2a, treated versus controls, p < 0.05; and Chart 2b: cyclophosphamide + MTP (20 $\mu$g) versus cyclophosphamide or MTP alone, p < 0.001.

and cyclophosphamide died of respiratory infection without the presence of tumor.

Tumor growth rate was followed up to Days 21 to 24 (Chart 1). Adriamycin and cis-platinum were ineffective in retarding tumor growth, whereas cyclophosphamide prevented tumor size increase after Day 14 at a $10^5$ tumor cell dose level and significantly retarded tumor growth when $10^6$ tumor cells were used for inoculation.

This experiment clearly established that, for Dunning prostatic carcinoma-inoculated animals, cyclophosphamide provided the greatest protection among the 3 agents used and that a tumor cell dose higher than $10^4$ cells and lower than $10^6$ cells could be sensitive to cyclophosphamide therapy without eliminating the tumor.

Combination of Cyclophosphamide and MTP Therapies. Animals inoculated with $5 \times 10^4$ prostatic tumor cells were subjected to cyclophosphamide and MTP therapies alone and in combination. The combination of cyclophosphamide plus MTP (20 $\mu$g i.p.) produced the lowest incidence of tumor take (60%), compared to 70% for MTP (20 $\mu$g) alone and 90% for cyclophosphamide alone (Chart 2b). The tumor developed in 100% of the animals of all other groups by Day 18 (Chart 2a). The only significant difference in tumor takes was between cyclophospha-
mide plus MTP (20 μg) and the controls.

The mean survival times of tumor-bearing animals subjected to various treatments were affected as follows: MTP (10 μg), 32.7 ± 2.5 (S.D.) (versus control, 30.3 ± 3.1; no significant difference); cyclophosphamide, 44.1 ± 4.7 (versus control, significant increase; p < 0.001); MTP (20 μg), 42.4 ± 4.4 (versus control, significant increase; p < 0.001); cyclophosphamide plus MTP (10 μg), 36.3 ± 2.7 (versus control, significant increase; p < 0.001); and cyclophosphamide plus MTP (20 μg), 57.4 ± 6.2 (versus control, significant increase; p < 0.001). For cyclophosphamide plus MTP (20 μg) versus cyclophosphamide or MTP (20 μg) alone, there was a significant increase in survival (p < 0.001).

All of the treated groups had a significantly lower tumor growth rate and lower tumor size compared to controls (Chart 2a). There was no significant difference between cyclophosphamide and MTP (20 μg) treatments given alone. However, the combination of cyclophosphamide plus MTP (20 μg) was the most effective treatment. This combination group showed a significantly smaller tumor size than did groups in which either of these agents was given alone (p < 0.001). MTP (20 μg) was significantly more effective in inhibiting tumor size increase than was MTP (10 μg) (p < 0.001).

The differences in the time taken for the tumor to reach 1 cm (Chart 2a) were found to be as follows for the various treatments that were instituted: treated groups (excluding 10 μg MTP alone, significantly longer (p < 0.001). MTP (20 μg) was significantly more effective in inhibiting tumor size increase than was MTP (10 μg) alone (p < 0.001). However, the combination of cyclophosphamide plus MTP (20 μg) was the most effective treatment. This combination group showed a significantly smaller tumor size than did groups in which either of these agents was given alone (p < 0.001). MTP (20 μg) was significantly more effective in inhibiting tumor size increase than was MTP (10 μg) alone (p < 0.001).

The incidence of metastasis was lowest (33%) in the group treated with a combination of cyclophosphamide plus MTP (10 μg). It was 40% for a combination of cyclophosphamide and MTP (20 μg). Metastases were found in 70% of the animals treated with either cyclophosphamide alone or MTP (20 μg) alone. All of the control animals had lung and lymph node metastasis. Only the combinations provided significant difference with the controls (p = 0.005 to 0.006).

**Chemotherapy and Chemoimmunotherapy of Bladder Carcinoma BT4.** The experiment carried out with Fischer rats bearing orthotopic BT4 can be divided into 2 parts. In the first part, doses of cyclophosphamide (60 μg/kg) and cis-platinum (10 mg/kg), used for Dunning tumor in Copenhagen x Fischer F1 hybrid rats, were administered alone and in combination with MTP (10 and 20 μg). These doses of cyclophosphamide and cis-platinum appeared to be toxic for Fischer rats, based on the following criteria: (a) the animals died carrying small tumors; and (b) the mean survival time, in some cases, was indeed shorter than that of the untreated control animals or those treated with MTP alone (Table 2).

Autopsies of animals that died at these higher doses of cyclophosphamide showed that, with cyclophosphamide alone, the animals (6 of 8) carried tumors, and 2 were "lost," having been eaten by the other rats overnight. On the other hand, 6 of 8 animals given cyclophosphamide plus MTP carried no tumor, and 2 were "lost" in this group as well.

Less dramatic results were obtained with cis-platinum; 1 animal was lost in the cis-platinum-alone group, and the rest (7 of 8) died carrying tumors, and 4 of these 7 carried small tumors. cis-Platinum combined with MTP obtained 30% tumor-free rats, and the others (7 of 10) carried islands of tumor cells surrounded by fibrotic tissue, as revealed on pathological examination of tissue sections.

In the second part of the experiment, the doses of cyclophosphamide and cis-platinum were lowered by one-half. The results of this experiment are presented in Table 2. It was observed that animals treated with MTP alone and cis-platinum alone had

---

**Table 2**

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Survival time (days)</th>
<th>Bladder wt (g)</th>
<th>No. of animals with apparent tumor/total</th>
<th>Metastasis to Lung</th>
<th>Lymph node</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no treatment)</td>
<td>43.33 ± 10.63</td>
<td>1.78 ± 0.69</td>
<td>0/8</td>
<td>6/9</td>
<td>4/9</td>
</tr>
<tr>
<td>Cy (60 mg/kg)</td>
<td>49.86 ± 13.64</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>Cy (60 mg/kg) + MTP (20 μg/day)</td>
<td>22.0 ± 13.29</td>
<td>3/10</td>
<td>0/10</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>cis-Platinum (10 mg/kg)</td>
<td>34.10 ± 19.60</td>
<td>1.11 ± 0.38</td>
<td>0/7</td>
<td>2/7</td>
<td>1/7</td>
</tr>
<tr>
<td>cis-Platinum (10 mg/kg) + MTP (20 μg/day)</td>
<td>0.18 ± 0.16</td>
<td>0/9</td>
<td>0/9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTP (20 μg/day)</td>
<td>0.056 ± 0.006</td>
<td>0/6</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis-Platinum (5 mg/kg)</td>
<td>0.38 ± 0.23</td>
<td>1.05 ± 0.38</td>
<td>0/8</td>
<td>1/8</td>
<td>1/8</td>
</tr>
<tr>
<td>cis-Platinum (5 mg/kg) + MTP (20 μg/day)</td>
<td>0.38 ± 0.23</td>
<td>1.05 ± 0.38</td>
<td>0/8</td>
<td>1/8</td>
<td>1/8</td>
</tr>
</tbody>
</table>

*Values are given for groups in which all of the animals died before termination of the experiment, i.e., 8 weeks after tumor inoculation. The animals died due to drug toxicity. There were no deaths in other treatment groups. These were sacrificed at the termination of the experiment, i.e., 8 weeks after tumor inoculation.

Bladder weight is given at the time of sacrifice, 8 weeks after tumor inoculation. For tumor size of animals that died due to drug toxicity, see text.

At the time of death or sacrifice.

Mean ± S.D.

Cy, cyclophosphamide.
smaller (1.05 to 1.11 g versus 1.78 g for controls) bladder weights (sacrificed 8 weeks after tumor inoculation), whereas cyclophosphamide treatment alone was the most effective in reducing bladder weight (0.18 g versus 1.78 g for controls). Combination of either cis-platinum or cyclophosphamide with MTP brought further diminution of bladder weight. The cis-platinum-plus-MTP combination gave bladder weights which were lower (0.38 g) than were the bladder weights of animals which received either of these treatments alone (1.05 to 1.11 g). Since cyclophosphamide alone was very effective in preventing bladder weight increase, the effect of MTP was dramatically demonstrated in 6 of 8 rats becoming free of their tumor and, in the other 2, the bladder weight was close to the weight of bladder in normal animals, and they carried only small foci of microscopic tumors. The incidence of metastasis was reduced in all of the treated animals, and no metastasis developed in animals treated with cyclophosphamide alone, cis-platinum plus MTP, and cyclophosphamide plus MTP.

Effect of Treatment of Bladder Tumors on Cellular Immunity. The animals in the above experiment were sacrificed 8 weeks after tumor inoculation, and their spleen cells were prepared and subjected to 3 assays of cellular immunity (Table 3). It was noted that spleen lymphocytes obtained from animals treated with cyclophosphamide and cyclophosphamide plus MTP gave 3.5- and 4.6-fold greater stimulation by phytohemagglutinin, respectively, than did the lymphocytes of control animals. On the other hand, cis-platinum treatment was ineffective, and combination with MTP was not able to recover the loss induced by cis-platinum. The macrophage migration inhibition assay indicated the presence of higher cellular immunity in all of the treatment groups, the major contribution being made by MTP alone, which was further augmented by cyclophosphamide or cis-platinum. The 51Cr release cytotoxicity assay performed with the spleen cells of cis-platinum-plus-MTP- and with cyclophosphamide-plus-MTP-treated animals demonstrated slight cytotoxicity (6.9 and 15.4%, respectively); the others exhibited no cytotoxicity at all.

**DISCUSSION**

The difficulty in early cancer detection has led to combined treatment strategies for the more advanced cancer cases. Which combinations would offer maximum promise with least discomfort is a difficult choice, since an increase in survival time often accompanies discomfort. New strategies must take into account the greater use of nontoxic agents to obtain longer survivals (23, 25). The present investigation was designed to test if nontoxic immunoadjuvant MTP could be used in conjunction with chemotherapeutic agents to obtain cures or longer survivals in tumor-bearing animals.

We used 2 transplantable tumor cell lines in this investigation. The first was Dunning R3327 A, a transplantable form of a prostatic carcinoma grown s.c. in Fischer × Copenhagen F1 hybrid rats, and the second tumor was a transplantable, well-differentiated transitional cell carcinoma of the bladder which was grown orthotopically (20) in syngeneic Fischer rats.

We chose 3 chemotherapeutic drugs that are commonly used against experimental and human prostatic carcinoma (11), namely Adriamycin, cis-platinum, and cyclophosphamide. A pilot study was first carried out to choose the most effective among them from the point of view of reducing tumor size, delaying tumor growth, and rendering animals tumor free when various small numbers of tumor cells were inoculated s.c. In the subsequent bladder tumor experiment, we chose cyclophosphamide, based on the results of the above pilot study, as well as cis-platinum, simply because cis-platinum is presently used in a number of centers in patients with advanced cancer of the bladder. As mentioned above, MTP was used as an agent because of a lack of toxicity and because of favorable results obtained in our previous studies (12, 25). In the present study, we have tested MTP immunotherapy alone, chemotherapy alone, and their combination.

In the case of prostatic carcinoma, the treatments were given 1 day after s.c. tumor inoculation, whereas, in the case of the bladder tumor, treatments were given 2 weeks after orthotopic tumor implantation.

The results of the pilot study indicated that cyclophosphamide was curative to animals that received 10⁶ cells. However, animals of this group died of infection, raising the possibility that the curative cyclophosphamide dose may also be toxic (immunosuppressive). In groups of animals receiving 10⁵ and 10⁶ tumor cells, where infection was absent, cyclophosphamide reduced tumor size and increased survival times significantly. In these experiments, we were unable to account for a lack of correlation between survival times and inoculated tumor cell dose in both control and treated animals. Only the tumor appearance times increased with increasing tumor cell dose. In the subsequent chemoimmunotherapy experiment, we used an intermediate dose (5 × 10⁴ cells) to enable us to study the effect of immuno-

---

**Table 3**

Effect of chemoimmunotherapy on the immune response of rats inoculated with BT4 bladder tumor

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Nonspecific immune response (phytohemagglutinin stimulation index)</th>
<th>Tumor-specific cellular immune response (%)</th>
<th>Specific 51Cr release</th>
<th>MMI index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>207</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MTP (20 μg/d)</td>
<td>355</td>
<td>0.0</td>
<td>37.0</td>
<td>58.3</td>
</tr>
<tr>
<td>Cy (30 mg/kg) + MTP (20 μg/day)</td>
<td>464</td>
<td>15.4</td>
<td>58.3</td>
<td></td>
</tr>
<tr>
<td>cis-platinum (5 mg/kg) + MTP</td>
<td>114</td>
<td>0.0</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td>(20 μg/d)</td>
<td>115</td>
<td>6.9</td>
<td>59.17</td>
<td></td>
</tr>
</tbody>
</table>

* Mean [3H]thymidine incorporation by phytohemagglutinin into spleen cells of treated animals × 100

**DISCUSSION**

The difficulty in early cancer detection has led to combined treatment strategies for the more advanced cancer cases. Which combinations would offer maximum promise with least discomfort is a difficult choice, since an increase in survival time often accompanies discomfort. New strategies must take into account the greater use of nontoxic agents to obtain longer survivals (23, 25). The present investigation was designed to test if nontoxic immunoadjuvant MTP could be used in conjunction with chemotherapeutic agents to obtain cures or longer survivals in tumor-bearing animals.

We used 2 transplantable tumor cell lines in this investigation. The first was Dunning R3327 A, a transplantable form of a prostatic carcinoma grown s.c. in Fischer × Copenhagen F1 hybrid rats, and the second tumor was a transplantable, well-differentiated transitional cell carcinoma of the bladder which was grown orthotopically (20) in syngeneic Fischer rats.

We chose 3 chemotherapeutic drugs that are commonly used against experimental and human prostatic carcinoma (11), namely Adriamycin, cis-platinum, and cyclophosphamide. A pilot study was first carried out to choose the most effective among them from the point of view of reducing tumor size, delaying tumor growth, and rendering animals tumor free when various small numbers of tumor cells were inoculated s.c. In the subsequent bladder tumor experiment, we chose cyclophosphamide, based on the results of the above pilot study, as well as cis-platinum, simply because cis-platinum is presently used in a number of centers in patients with advanced cancer of the bladder. As mentioned above, MTP was used as an agent because of a lack of toxicity and because of favorable results obtained in our previous studies (12, 25). In the present study, we have tested MTP immunotherapy alone, chemotherapy alone, and their combination.

In the case of prostatic carcinoma, the treatments were given 1 day after s.c. tumor inoculation, whereas, in the case of the bladder tumor, treatments were given 2 weeks after orthotopic tumor implantation.

The results of the pilot study indicated that cyclophosphamide was curative to animals that received 10⁶ cells. However, animals of this group died of infection, raising the possibility that the curative cyclophosphamide dose may also be toxic (immunosuppressive). In groups of animals receiving 10⁵ and 10⁶ tumor cells, where infection was absent, cyclophosphamide reduced tumor size and increased survival times significantly. In these experiments, we were unable to account for a lack of correlation between survival times and inoculated tumor cell dose in both control and treated animals. Only the tumor appearance times increased with increasing tumor cell dose. In the subsequent chemoimmunotherapy experiment, we used an intermediate dose (5 × 10⁴ cells) to enable us to study the effect of immuno-
therapy when used with partially effective cyclophosphamide chemotherapy. It was found that a combination of cyclophosphamide with MTP was more effective than was either of these given alone with respect to tumor size, inhibition of tumor growth, delay in tumor take, prolongation of the survival time, and lowering in the incidence of lung and lymph node metastases (Table 1; Chart 2).

In the case of bladder carcinoma, where we used inbred Fischer rats, it was noted that animals were more sensitive to the toxic effects of cyclophosphamide (60 mg/kg) and cis-platinum (10 mg/kg) than were Fischer × Copenhagen F1 hybrids (weight loss and early death). A high dose of either cyclophosphamide (60 mg/kg) or cis-platinum (10 mg/kg) plus MTP rendered the animals tumor free in 75 and 30% of rats, respectively, but, at the same time, these doses of cyclophosphamide and cis-platinum led to death of the animals earlier than they led to death of the controls. Smaller cis-platinum dose (5 mg) alone or with MTP did not destroy the tumor completely, but the combination reduced bladder weight significantly. A low dose of cyclophosphamide (30 mg/kg) alone was effective in reducing bladder weight but, when combined with MTP, it obtained 75% tumor-free animals. The rest of the tumor-bearing rats had small foci of bladder tumors.

Having obtained a treatment regimen that rendered animals tumor free, we studied the mechanism of antitumor action using parameters of cell-mediated immunity. We utilized: (a) blastogenic response of spleen lymphocytes to the nonspecific mitogen phytohemagglutinin. This test was done to measure the lymphoproliferative response following the administration of immunotherapeutic agents with B. Calmette-Guérin, C. parvum, or aniline mustards to eradicate tumor from plasmacytoma-bearing animals. The animals recovered from depression only after a period of 2 weeks that elapsed between termination of the treatment and termination of the toxic effects of cyclophosphamide (60 mg/kg) and c/s-platinum. The lymphocytes of other groups of animals showed no cytotoxicity, which may either be due to the large tumor size in the animals, as shown by O’Toole et al. (26) and Catalona and Chretien (6), or to the slow ability of lymphocytes to recover from the suppressive effects of chemotherapy, as suggested by Padarathsingh et al. (27).

Padarathsingh et al. (27) also observed severe depression of the lymphoproliferative response following the administration of aniline mustards to eradicate tumor from plasmacytoma-bearing animals. The animals recovered from depression only after a prolonged period of 4 to 6 weeks. Administration of an immunoadjuvant (levamisole) following aniline mustard had a significant effect on the ability of mice to resist tumor challenge and the ability of immune lymphocytes to affect tumor cell neutralization in tumorigenic experiments.

Several studies have been carried out which combine chemotherapeutic agents with B. Calmette-Guérin, C. parvum, or levamisole. They indicate that the combinations are superior to any of the agents given alone (8, 9, 16, 17, 21, 22). These findings were confirmed recently by Vanhaelen and Fisher (31), who demonstrated a true synergism between C. parvum and 2 cytostatic agents, namely cyclophosphamide and cis-platinum. Although neither of the cytostatic agents or the immunostimulant given alone was able to prevent the progression of disease in their tumor model (mouse ovarian teratocarcinoma), C. parvum combined with cyclophosphamide or cis-platinum yielded 40 and 60% cures, respectively. Similar synergism of C. parvum with alkylating agents and antimetabolites has been described in a murine model of breast cancer (13, 14). In our model of bladder carcinoma of the rats, the cure rate, as judged by the absence of apparent tumor or tumor metastasis, was 75% in the combination of cyclophosphamide and MTP. While the combination of cis-platinum with MTP was not curative, their actions were synergistic.

It is apparent that chemotherapy alone was unable to kill the last tumor cell in our experiment, as well as those described by Mathé (24), even if the drug dose was maximally increased. However, giving MTP as an immunoadjuvant in addition to the chemotherapy appeared to allow the immune system to deal with the residual cancer cells and obtain some animals without apparent tumor.

REFERENCES

15. Glaser, M. Regulation of specific cell-mediated cytotoxic response against SV-40-induced tumor-associated antigens by depletion of suppressor T-cells with
Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22-24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 821; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O2 consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = +0.27 and L = -0.16 and the normal differential is 65 per cent M and 35 per cent L, then

\[ 0.65 (+0.27) + 0.35 (-0.16) = +0.12 \]

a figure identical to the observed +0.12 for normal leukocytes.
Combined Cyclophosphamide Chemotherapy and Maltose Tetrapalmitate Immunotherapy in the Treatment of Transplanted Bladder and Prostate Carcinoma of the Rat


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/44/2/536

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.