Host-mediated Therapeutic Effects Produced by Appropriately Timed Administration of Bleomycin on a Rat Fibrosarcoma

Kiyoshi Morikawa, Masuo Hosokawa, Jun-ichi Hamada, Michio Sugawara, and Hiroshi Kobayashi

ABSTRACT

The timing of bleomycin (BLM) administration after KMT-17 tumor inoculation was found to be important for optimizing its therapeutic effect on tumor-bearing rats. A remarkable therapeutic effect was observed when BLM (5 mg/kg/day) was administered i.p. for 5 days from the eighth day after tumor inoculation (Day 8 to Day 12) rather than when BLM was administered i.p. for 5 days during the days immediately following tumor inoculation (Day 1 and Day 5) (cured rats/treated rats: 10/21 and 2/16, respectively). By means of a Winn assay, stronger tumor-neutralizing activities were observed in spleen cells from BLM (Day 8 to Day 12)-treated tumor-bearing rats than were observed in spleen cells from BLM (Day 1 to Day 5)-treated tumor-bearing rats (% Inhibition: 70.9 and 49.3%, respectively). These therapeutic effects were thus found to be consistent with the antitumor immunity against KMT-17. The enhanced tumor-neutralizing activities of spleen cells from BLM-treated tumor-bearing rats were suppressed by adding spleen cells from nontreated tumor-bearing rats. In cell transfer experiments, an antitumor transplanation mechanism was observed in rats immunized with irradiated KMT-17 cells which was abrogated by an adoptive transfer of spleen cells from untreated tumor-bearing rats or BLM (Day 1 to Day 5)-treated tumor-bearing rats but not from BLM (Day 8 to Day 12)-treated tumor-bearing rats. These results suggest that, when BLM is administered during a late stage of tumor growth, it is effective in eliminating suppressor cells and that this leads to an improvement in the therapeutic effects of the drug.

INTRODUCTION

Most of the antitumor drugs used in cancer chemotherapy are known to induce immunosuppression as a side effect. Several reports, however, have demonstrated that antitumor drugs may cause an enhancement of antitumor immune responses. These indicate that the antitumor immunity enhanced by the drugs is able to facilitate the therapeutic effects of the drugs. The intensity of such host-mediated therapeutic effects of the drugs depends upon the dose and the regimen. For instance, the injection of a low dose of Cy\(^2\) at a late stage of tumor growth leads to an increase in antitumor immunity and cures most large tumor-bearing hosts, but the injection of the drug at an early stage does not. It has been suggested that the mechanism responsible for the enhancing effect of the drug eliminates the suppressor cell activity that interferes with the generation of potent antitumor immunity (5, 9, 14, 15, 21, 24).

Using our experimental model of the WKA rat and its syngeneic KMT-17 tumor, we have shown previously that an antileukemia drug, busulfan, causes a time-dependent augmentation of antitumor transplantation resistance in rats immunized with X-irradiated tumor cells and that this may be related to the elimination of suppressor cell activity (10, 11, 19). Using the same tumor models, we have now attempted to determine whether the immunopotentiating effects of antitumor drugs are able to reflect the therapeutic effects. An antitumor antibiotic, BLM, was selected for use in the present study, since the drug has shown little or no immunosuppressive activity (1, 17, 23) or immunostimulating activity under strictly defined conditions (18). In addition, BLM is used widely in clinical oncology.

MATERIALS AND METHODS

Rats. The rats used were 8- to 12-week-old female inbred Wistar King ApteKman/Hok (WKA) rats supplied by the Institute of Experimental Animals, Hokkaido University School of Medicine, Sapporo, Japan. Tumor. KMT-17 is a methylcholanthrene-induced fibrosarcoma in WKA rats. The KMT-17 was maintained in an ascites form by a passage every 3 days. The cell doses of KMT-17 which caused 50% lethality when injected s.c. into WKA rats were 5 x 10\(^5\).

Tumor-bearing Rats. WKA rats were given inoculations s.c. in the back with 1 x 10\(^5\) viable KMT-17 cells. After inoculation of the tumors, rats were randomly assigned to experimental and control groups.

Bleomycin. The BLM, which was supplied by Nippon Kayaku Co., Ltd., Tokyo, Japan, was dissolved with sterile 0.85% NaCl solution and administered i.p. once a day for 5 days according to an agreed-upon time schedule.

Preparation of Spleen Cell Suspensions. Spleens that had been aseptically removed from rats were teased into loose-fitting glass homogenizers. The cell suspensions were passed through 4 layers of gauze and washed twice with cold Eagle's minimum essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). The number of viable cells was determined by a trypan blue exclusion test.

In Vivo Tumor-neutralizing Assay (Winn Assay). A modified form of the Winn assay (22) was performed as described previously (11). A spleen cell suspension (5 x 10\(^5\) in 0.1 ml) was mixed with an equal volume of 1 x 10\(^5\) tumor cells, and 0.2 ml of the mixture was inoculated s.c. in syngeneic rats which had been irradiated with 400 rads before inoculation and immediately thereafter rescued with 5 x 10\(^5\) bone marrow cells. Eleven days after inoculation, hosts were sacrificed, and tumor weights were measured. In the abrogation experiment, spleen cells (5 x 10\(^5\) in 0.1 ml) obtained from nontreated tumor-bearing rats were added to the mixture of spleen cells (5 x 10\(^5\) in 0.1 ml) obtained from BLM-treated tumor-bearing rats and KMT-17 cells (1 x 10\(^5\) in 0.1 ml). The following procedure has already been described above. The inhibition of tumor growth was calculated by a formula in which a was the mean tumor weight in rats inoculated with tumor cells alone and b was the mean tumor weight in rats inoculated with the mixture of spleen cells and tumor cells.

\[
\% \text{ of inhibition} = \frac{a - b}{a} \times 100
\]
HOST-MEDIATED THERAPEUTIC EFFECTS OF BLM

Adoptive Transfer of Experiments. Two hundred million spleen cells from (a) nontreated tumor-bearing rats, (b) tumor-bearing rats which were treated with BLM for 5 days from Day 1 to Day 5, and (c) tumor-bearing rats which were treated with BLM for 5 days from Day 8 to Day 12 were adoptively transferred i.v. into syngeneic rats, which had been immunized intradermally with X-irradiated (8000 rads) KMT-17 cells, 9 days after the immunization. One hundred thousand KMT-17 cells were inoculated s.c. in immune rats 10 days after the immunization.

Statistical Analysis. A significant difference in survival rate was calculated by a χ² test, and the difference in tumor weight was calculated by a Student's t test.

RESULTS

Dose Dependency of the Therapeutic Effect on KMT-17 of BLM. Rats were inoculated s.c. with KMT-17 cells (1 x 10⁵) on Day 0 and were then treated with BLM at various doses ranging from 0.63 to 20 mg/kg/day for 5 days from Day 8 (Table 1). Treatment of rats given 1.25 mg/kg/day resulted in a cure rate of only 20%, whereas treatment of rats given 2.5 or 5.0 mg/kg/day resulted in a cure rate of about one-half (60 and 48%, respectively). Treatment of rats given 10 or 20 mg/kg/day cured none of the rats and shortened the survival time of the remainder when compared to nontreated rats. The treatment seemed to cause toxicity. In further experiments, we have treated tumor-bearing rats with BLM at the dose of 5 mg/kg/day for 5 days (total dose, 25 mg/kg).

Timing Dependency of the Therapeutic Effect on KMT-17 of BLM. In order to examine the timing dependency of the therapeutic effect of BLM, tumor-bearing rats were treated with BLM (5 mg/kg/day) for 5 days from Days 1, 6, 8, and 11 (Table 2). BLM therapy commencing on Day 1, when the tumor was not palpable, gave only a 13% rate of cured rats, whereas BLM therapy from Day 8, when the tumor size was about 15 mm in diameter, gave a cure rate of 48%. BLM therapy from Day 11 cured none of the rats. As is shown in Chart 1, when BLM was administered from Day 1, the growth of tumors was inhibited initially when compared with that of nontreated rats, but tumors continued to grow and eventually killed most of the rats. On the other hand, when BLM was administered from Day 8, an initial tumor growth was observed, but it thereafter regressed. These results suggest that the effect of BLM does not depend on the tumoricidal activity of the drug alone.

The combined early (Day 1 to Day 5) and late (day 8 to Day 12) treatment of BLM (total dose 50 mg/kg) gave a cure rate of 85% (Table 2). Rats cured by BLM therapy were challenged with 1 x 10⁶ KMT-17 tumor cells again (Table 3). All the rats cured by the combined early and late administration of BLM were also able to reject the new tumor challenge, as were the rats cured by the late BLM treatment. These results indicate that the early treatment of BLM does not interfere with the effect of BLM administered again at a late stage.

Enhancement of Tumor-neutralizing Activity of Spleen Cells from Tumor-bearing Rats by BLM Therapy during a Late Stage of Tumor Growth. Correlation of the therapeutic effects of BLM and enhanced antitumor immunity was examined by an

<table>
<thead>
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<th>Table 2</th>
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<tr>
<td>Timing dependency of therapeutic effects of BLM on KMT-17 tumor in rats</td>
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<tr>
<td>KMT-17 cells (1 x 10⁵) were implanted s.c. in rats on Day 0, and BLM (5 mg/kg/day) was administered i.p. for 5 days (Experiment 1) or 10 days (Experiment 2) on indicated days.</td>
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<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treated with BLM on Days</th>
<th>Cured rats/treated rats</th>
<th>MSD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1–5</td>
<td>2/16⁎ (13%)</td>
<td>22.1 ± 5.1⁎</td>
</tr>
<tr>
<td>6–10</td>
<td>3/9 (33%)</td>
<td>19.7 ± 7.4</td>
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<tr>
<td>8–12</td>
<td>10/21 (48%)</td>
<td>31.2 ± 7.7⁎</td>
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</tr>
<tr>
<td>11–15</td>
<td>0/6 (0%)</td>
<td>16.5 ± 4.8</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0/6 (0%)</td>
<td>22.9 ± 5.8⁹</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1–5, 8–12</td>
<td>11/13 (85%)</td>
<td>37.0 ± 5.9</td>
</tr>
<tr>
<td>None</td>
<td>0/13 (0%)</td>
<td>22.3 ± 5.4</td>
<td></td>
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* MSD, mean survival days.  
† Numbers in parentheses, percentage.  
9 Mean ± SD.  
‡ Significantly different (d: P < 0.05; e to j: P < 0.01).
in vitro-in vivo tumor-neutralizing assay (Winn assay), using spleen cells as effector cells. As is shown in Table 4, the tumor-neutralizing activity was found in spleen cells obtained from nontreated tumor-bearing rats treated with BLM during a late stage of tumor growth (Day 8 to Day 12) (70.9%). On the other hand, the tumor-neutralizing activity of spleen cells from tumor-bearing rats treated with BLM during an early stage (Day 1 to Day 5) was not augmented (49.3%). The therapeutic effect of BLM thus correlated well with the antitumor immunity of the hosts. As can be seen in Table 5, the enhanced antitumor immunity of BLM-treated rats continued until a late stage of tumor growth (Day 18) (56.0%). However, no tumor-neutralizing activity was found in spleen cells obtained from nontreated tumor-bearing rats on Day 18 (12.0%). These results suggest that the therapeutic effects of BLM depend on its immunological effects.

Elimination of Suppressor Cell Activity Detected in Tumor-Bearing Rats by Effectively Timed BLM Treatment. A mixture of $1 \times 10^5$ KMT-17 cells and $5 \times 10^6$ spleen cells from BLM (Day 8 to Day 12)-treated rats was added to $5 \times 10^6$ spleen cells from nontreated rats and was then inoculated s.c. into irradiated syngeneic rats. As is shown in Table 6, spleen cells from BLM-treated rats on Day 13 inhibited significantly the growth of KMT-17 (% of inhibition, 86.5%). However, the neutralizing activity of spleen cells from BLM-treated rats was abrogated by the addition of spleen cells from nontreated tumor-bearing rats on Day 13 or Day 18 but not of spleen cells from normal rats (68.2, 50.1, and 91.2%, respectively). These results suggest that the enhancement of antitumor immunity by BLM treatment is due to the selective elimination of suppressor cell activity. This hypothesis was further supported by adoptive transfer experiments. As is shown in Table 7, when rats were immunized i.d. with X-irradiated KMT-17 cells, only 2 of 16 rats died from challenged KMT-17. The antitumor transplantation resistance induced in the immune rats was abrogated by the adoptive transfer of $2 \times 10^8$ spleen cells from nontumor-bearing rats or BLM (Day 1 to Day 12)-treated tumor-bearing rats (the lethal growth of KMT-17 was 8/15 and 5/10, respectively).
tively). On the other hand, the antitumor resistance was not abrogated by the adoptive transfer of spleen cells from BLM (Day 8 to Day 12)-treated tumor-bearing rats (0/11).

**DISCUSSION**

The findings presented in this paper reveal that the therapeutic effects of BLM depend not only on the direct tumoricidal activity of the drug but also on its immunoaugmenting effect. The therapeutic effect of BLM was more marked in rats treated from Day 8, when the mean tumor size was 15 mm in diameter, than in rats treated from Day 1, when the tumor was not palpable (Table 2, Chart 1). The timing-dependent therapeutic effect of BLM on KMT-17 correlated well with the antitumor activity shown by the results of Winn assay (Table 4). The tumor-neutralizing activity of spleen cells was significantly enhanced by the treatment with BLM during the late stage but not by the treatment during the early stage. It is now important to consider whether the enhanced antitumor immunity is due merely to the results of tumor regression brought about by the chemotherapy. This is unlikely, however, because the tumor burden in the effective group had not yet differed from that in the ineffective group on Day 13 when spleen cells were taken for the Winn assay (Chart 1). We can speculate, therefore, that the enhanced antitumor immunity affects the tumor growth thereafter or prevents the development of dormant tumor foci. Other evidence suggesting the immunological effect of BLM treatment is that all the cured rats acquired complete resistance against rechallenge by the tumor (Table 3).

It has been reported that the antitumor immune response of KMT-17 tumor-bearing rats can be detected from Day 8 and reaches a peak on Day 12 (12). This stage corresponded well to our own appropriate administration timing of BLM. We therefore suggest that BLM is able to enhance antitumor response at the time when a sufficient level of antitumor immunity is developing in the tumor-bearing rats. The BLM-enhanced antitumor activity of spleen cells was abrogated by the admixture with spleen cells from nontreated tumor-bearing rats (Table 6), while the antitumor resistance induced by the immunization with irradiated KMT-17 cells was abrogated by the adoptive transfer of spleen cells from nontreated rats or rats treated with BLM during the early stage but not from rats treated with the drug during the late stage (Table 7). These findings suggest that the appropriately timed administration of BLM eliminates suppressor cell activity that is detectable in tumor-bearing rats.

At the present time, the nature of BLM-sensitive suppressor cells described here is still unknown. Our data show that an appropriately timed administration of BLM before immunization with irradiated KMT-17 tumor results in the augmentation of antitumor transplantation resistance. One may speculate that the mechanism of BLM action operates differently on suppressor cell activity in immune rats and tumor-bearing rats. BLM, which affects suppressor precursors in immune rats, may affect antigen-differentiated suppressor cells in tumor-bearing rats if administered during the late stage.

Thus far, only a few reports have been published concerning the importance of the timing of chemotherapy. It has been reported that CY therapy given to mice bearing large tumors at a late stage of tumor growth is more effective for curing the mice than is therapy given to mice bearing nonpalpable tumors at an early stage (6, 13) and that tumor regression in mice bearing large tumors is caused by enhanced antitumor immunity through the elimination of suppressor cell activity by the drug (7, 14, 24). This timing dependency of the CY therapy treatment of mice is similar to that of the BLM therapy treatment of rats. There is a difference, however, in the effective dose of the 2 drugs for the enhancement of antitumor immunity. Antitumor immunity is enhanced by the administration of a relatively high dose of BLM (total dose 25 mg/kg) which is almost equal to the dose used in the ordinary chemotherapy applied to rats. On the other hand, in order for CY to enhance the antitumor response, it has to be administered at a low dose, as a high dose of CY suppresses immune responses because it eliminates not only suppressor cells but also the precursors of antitumor effector cells (6, 13). BLM therapy of rats during the late stage is even effective in rats that have been treated with BLM during the early stage (Table 2). Following this combined early and late administration of the drug, antitumor resistance can be detected in the cured rats (Table 3). On the other hand, the combined early and late administration of CY is not effective (6). This difference may be due to the fact that BLM, being different than CY, is characterized by little or no causation of immunosuppressive activity (1, 17, 23). One may speculate, therefore, that the effect of a high dose of BLM is selective as to suppressor cell activity but is not generally immunosuppressive. It is an attractive feature of the drug that a relatively high dose of BLM augments antitumor immunity and that these host-mediated therapeutic effects of the drug cooperate with the direct tumoricidal activity.

Other possibilities for the mechanism of host antitumor immunity enhancement by BLM may be offered: (a) the activity of antitumor effector cells may be enhanced by the drug (8, 16, 20). This is supported by preliminary data which suggest that BLM enhances the cytolytic activity of peritoneal macrophage in WKA rats; (b) the production of immunostimulating cytokines such as interleukin 2 may be enhanced by the drug (2, 18); and (c) tumor cells may be rendered more immunogenic by the drug (3, 4). Further studies will therefore be necessary to investigate the action of BLM on antitumor immunity in tumor-bearing hosts, and these possibilities are currently under further investigation in our laboratory.

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