Enhancement of Antitumor Transplantation Resistance in Rats by Appropriately Timed Administration of Busulfan

Yutaka Mizushima, Fujiro Sendo, Noritoshi Takeichi, Masuo Hosohawa, and Hiroshi Kobayashi

ABSTRACT
Enhancement of specific transplantation resistance to a syngeneic tumor (KMT-17) was observed in WKA rats by treatment with the antileukemia drug busulfan (BU) (15 mg/kg) 5 days before and 5 days after immunization with X-irradiated KMT-17 tumor cells. Rats immunized with X-irradiated KMT-17 cells and then treated with BU showed specific transplantation resistance only against KMT-17 tumor. Carrageenan administration after BU treatment had no effect on enhancement by BU, which indicated that macrophages were not playing a major role in the observed enhancement. With the Winn assay, it was found that spleen cells from rats immunized with X-irradiated tumor cells followed by BU inhibited the growth of admixed tumor cells more strongly than did spleen cells from rats only immunized or only BU treated and that the tumor-neutralizing activity of spleen cells from rats treated by immunization followed by BU was abrogated by treatment with anti-T-serum and complement. It was suggested that the enhanced antitumor transplantation resistance caused by BU was due to enhanced T-cell-mediated immune responses to tumor cells. Enhancement of antitumor transplantation resistance by BU was significantly abrogated by adoptive transfer with thymus cells and was slightly abrogated with spleen cells from rats immunized with X-irradiated KMT-17 cells 1 day before tumor challenge but receiving no other treatment. Transfer of sera from the immunized rats had no effect on enhancement by BU. These results, taken together, suggest that the mechanism of the enhancement by BU involved a selective elimination of the immunosuppressor cells from the immunized hosts.

INTRODUCTION
In a previous paper (17), we reported that a transplanted syngeneic tumor (KMT-17) spontaneously regressed in WKA rats pretreated with the antileukemia drug BU. In further experiments, we demonstrated (13) that tumor regression in BU-pretreated rats was caused by the enhancement of T-cell-mediated immune responses to tumor cells and that this immune enhancement by BU pretreatment was due to the selective elimination of immunosuppressor cells.

In order to learn in more detail the effects of BU on the immune responses of hosts, we examined its effects on the antitumor transplantation resistance induced by immunization with X-irradiated tumor cells, comparing such results with those of other anticancer drugs. In this paper, enhancement of antitumor transplantation resistance was demonstrated in WKA rats by treatment with BU either before or after immunization with X-irradiated tumor cells, and the mechanisms of that enhancement by BU were studied.

MATERIALS AND METHODS
Rat
Inbred Wistar-King-Aptekman/Hok (WKA) rats were obtained from the Institute of Experimental Animals, Hokkaido University School of Medicine, Sapporo, Japan.

Tumors
KMT-17 is a 3-methylcholanthrene-induced fibrosarcoma in a WKA rat. WFT-2N is a lymphoma subline established from the leukemia spleen cells of a WKA rat given an injection of Friend leukemia virus. The cell doses of KMT-17 and WFT-2N causing 50% lethality when injected s.c. into WKA rats were 5 x 10^3 and 5 x 10^2, respectively. These tumors were maintained in ascites form.

Busulfan
The BU used was Mablin powder (Takeda Pharm. Co., Ltd., Osaka, Japan) dissolved in sugar syrup and given i.g. by means of a Nelaton catheter.

Carrageenan
Carrageenan (Sigma Chemical Company, St. Louis, Mo.) was dissolved in phosphate-buffered saline, sterilized by autoclaving at 100° for 15 min, and injected i.p. at a dose of 5 mg/rat.

Immunization
One hundred million X-irradiated (8000 rads) tumor cells were injected once s.c. into the right flanks of the rats.

Standard Schedule of Treatment
WKA rats were immunized with 1 x 10^6 X-irradiated KMT-17 cells on Day 0. BU, 15 mg/kg, was given either 5 days before or 5 days after the immunization. One hundred thousand KMT-17 cells were injected s.c. on Day 10 or 20.

Preparation of Cell Suspensions
Spleens and thymuses were aseptically removed from the animals and teased in loosely fitting glass homogenizers. The cell suspensions were passed through gauze and washed twice.
in Eagle’s minimal essential medium, and the number of viable cells was determined by trypan blue dye exclusion.

**Treatment of Spleen Cells with Anti-T-Serum**

Spleen cells were treated with anti-T-serum and fresh guinea pig complement for 45 min at 37° in a rocker water bath. About 45% of WKA rat spleen cells were killed. Rabbit anti-rat T-serum was prepared as described by Ishii et al. (7). Briefly, T-cells were purified from the mesenteric lymph nodes of the WKA rats by nylon fiber column filtration and were injected i.v. into rabbits. These animals were boosted 3 times at 1-week intervals and bled 1 week later. The sera were absorbed with KMT-17 and syngeneic bone marrow cells. The anti-rat T-serum specificity was examined by F. Sendo. Treatment of the WKA rat spleen cells with this anti-rat T-serum and guinea pig complement abrogated the blastogenic response to concanavalin A, did not affect the response to lipopolysaccharide, and slightly affected the natural killer activity of the spleen cells.4

**In Vivo Tumor Neutralization Assay (Winn Assay)**

A modified form of the Winn assay (25) was used. Spleen cell suspension (1 x 10⁷; 0.1 ml) was mixed with an equal volume of 1 x 10⁹ tumor cells, with 0.2 ml of the cell mixture being injected s.c. into syngeneic rats irradiated with 350 rads and rescued with 1 x 10⁷ bone marrow cells 1 day before inoculation. Tumor size (the mean of 2 dimension diameters) was serially measured.

**Transfer Experiments**

**Serum Transfer.** Serum (1 ml) obtained from rats 6, 7, and 10 days after immunization with X-irradiated KMT-17 cells was transferred i.v. on Days 6, 8, and 10, respectively, into rats immunized on Day 0 followed by BU on Day 5. One hundred thousand KMT-17 tumor cells were injected s.c. on Day 10.

**Cell Transfer.** Four hundred million thymus cells or 2 x 10⁸ spleen cells (from normal rats, from rats 9 days after immunization, or from rats 9 days after immunization followed by BU on Day 5) were adoptively transferred i.v. on Day 9 into WKA rats immunized with X-irradiated KMT-17 tumor cells on Day 0, followed by BU on Day 5. One hundred thousand KMT-17 tumor cells were injected s.c. on Day 10.

**Statistical Analysis**

The significant difference in mean survival time was calculated by Student’s t test, and the difference in survival rate was calculated by Fisher’s exact test.

**RESULTS**

**Effects of BU on Antitumor Transplantation Resistance Induced by Immunization with X-irradiated Tumor Cells**

To examine the effects of BU on the antitumor transplantation resistance induced by immunization with 1 x 10⁸ X-irradiated KMT-17 tumor cells, BU, 15 mg/kg, was given to WKA rats either 5 days before or 5 days after immunization, and 1 x 10⁶ KMT-17 cells were then injected s.c. 10 or 20 days after immunization. As shown in Table 1, when 1 x 10⁶ KMT-17 tumor cells were injected 10 days after immunization, the survival rate in the group receiving immunization alone was 14.3%, a very low percentage. However, when BU was given 5 days after immunization, the survival rate increased to 80.0%. This was significantly higher than the combined rate (48.8%) of the results in the group receiving immunization alone (14.3%) and those in the group receiving BU treatment alone (34.5%).

When BU was given 5 days before immunization, the survival rate significantly increased to 53.3%, and the survival time was also significantly prolonged in comparison with the group receiving immunization alone. Thus, transplantation resistance was enhanced by BU treatment. Enhancement of antitumor transplantation resistance was also observed when tumor cells were injected s.c. 20 days after immunization. As previously described by Sendo et al. (17), the spontaneous regression of KMT-17 tumor by BU pretreatment was caused by the immunological mechanisms, not by the direct cytotoxic effect of BU against the tumor cells injected. Since the effect of BU post-treatment on the induction of antitumor transplantation resistance was synergistic and the effect of BU pretreatment was additive, the following experiments were performed according to the schedule described below: immunization on Day 0; BU treatment on Day 5; and tumor challenge on Day 10.

To examine the effects of the doses of BU on enhancement, BU, 5, 15, and 20 mg/kg, was applied. No significant enhancement was observed when BU, 5 mg/kg, was given, while some rats given BU, 20 mg/kg, died of severe bone marrow suppression (data not shown). Therefore, BU, 15 mg/kg, was used in this study.

To ascertain whether rats which had rejected the first injection of 1 x 10⁶ KMT-17 cells acquired transplantation resistance to the identical tumor, 1 x 10⁶ KMT-17 cells were reinjected s.c. 6 weeks after the first injection. Rats rejecting the first injection all rejected the second injection of tumor cells. To examine the specificity of antitumor transplantation resistance, WKA rats immunized with X-irradiated KMT-17 tumor cells, BU, 15 mg/kg, was given p.o. either 5 days before or 5 days after immunization, and 1 x 10⁶ KMT-17 cells were injected s.c. on Day 10 or 20. These results were the total of several experiments.

**Table 1**

<table>
<thead>
<tr>
<th>Immunization on Day 0</th>
<th>BU on Day</th>
<th>Challenged on Day 10</th>
<th>Survivors/rats used (%)</th>
<th>Mean survival time (days)</th>
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<td>Yes +5</td>
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<td>32.0 ± 2.6</td>
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<td>5/35 (14.3)</td>
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<td>19.8 ± 0.9</td>
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<td>9/11 (81.8)</td>
<td>(26, 32)</td>
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<tr>
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<td>5/12 (41.7)</td>
<td>35.4 ± 6.4</td>
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<td>20</td>
<td>0/10 (0)</td>
<td>18.3 ± 0.5</td>
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</table>

*<0.02 compared with the group receiving immunization alone.

**Statistical Analysis**

The significant difference in mean survival time was calculated by Student’s t test, and the difference in survival rate was calculated by Fisher’s exact test.

**RESULTS**

**Effects of BU on Antitumor Transplantation Resistance Induced by Immunization with X-irradiated Tumor Cells**

To examine the effects of BU on the antitumor transplantation resistance induced by immunization with 1 x 10⁷ X-irradiated
cells followed by BU were given s.c. injections of WFT-2N tumor, which was an immunogenic lymphoma antigenically different from KMT-17 (17). As shown in Table 2, rats immunized with KMT-17 cells followed by BU did not show any transplantation resistance to the WFT-2N tumor. These results suggest that the specific transplantation resistance to KMT-17 tumor was enhanced by BU treatment.

**Immunological Studies on the Mechanisms of Enhancement of Antitumor Transplantation Resistance by BU**

In order to determine the mechanisms of enhancement of antitumor transplantation resistance by BU treatment, the following experiments were performed.

**Carrageenan Treatment.** To examine whether enhanced antitumor transplantation resistance by BU was caused by the enhanced macrophage tumoricidal function as previously reported by Schultz et al. (15) and Stoychkov (20), carrageenan, 5 mg/rat, was given twice i.p. on Days 6 and 7 to WKA rats immunized with X-irradiated tumor cells on Day 0, followed by BU on Day 5. Concerning the effective dose of carrageenan for inhibiting the functions of macrophages, Kodama in our laboratory used carrageenan, 5 mg/rat, as Keller (10) has already reported, and checked its effects on the incidence of pulmonary metastasis and on the carbon clearance. He demonstrated that a single i.p. 5-mg/rat injection of carrageenan into normal WKA rats 2 days before i.v. injection of KMT-17 cells enhanced pulmonary nodule formation and that adoptive transfer of alveolar macrophages from normal rats into carrageenan-treated rats reduced the incidence of pulmonary metastasis to the level found in untreated rats. He also demonstrated with a carbon clearance test that a single 5-mg/rat injection of carrageenan was sufficient for inhibition of phagocytosis. We administered carrageenan, 5 mg/rat, twice i.p. in this experiment. Therefore, the dose of carrageenan used in this experiment seems adequate to inhibit the functions of macrophages. As shown in Table 3, carrageenan treatment did not affect the enhancement of antitumor transplantation resistance by BU. This result suggests that the enhanced antitumor transplantation resistance caused by BU was not mediated by the enhanced macrophage cytotoxic function.

**In Vivo Winn Assay.** In order to clarify that the enhanced antitumor transplantation resistance caused by BU was due to the enhanced immune responses to tumor cells as well as to determine the main effector cells operating in the enhancement by BU, the in vivo Winn assay was used. As shown in Table 4, spleen cells from rats immunized with X-irradiated tumor cells on Day 0 followed by BU on Day 5 inhibited the growth of admixed tumor cells more strongly than did those from rats receiving immunization alone. Spleen cells from rats treated by BU without immunization (Group 3) showed no tumor-neutralizing activity. To determine whether the main effector cells in the observed tumor inhibition were T-cells, the effects of anti-T-serum on the tumor-neutralizing activity of the spleen cells were examined. As shown in Table 5, the tumor-neutralizing activity of the spleen cells from rats first immunized and then receiving BU was significantly abrogated by treatment with anti-T-serum and complement. These results suggest that the enhancement of antitumor transplantation resistance by BU was caused by the enhanced immune responses to tumor cells and that the main effector cells in the observed system belong to the T-cell population, although it is possible that some other cells, i.e., recipient’s macrophages, may influence the activity of sensitized T-cells.

**Transfer Experiments.** In order to learn whether enhancement of antitumor transplantation resistance by BU was due to the fact that BU inhibited the production of humoral blocking factors or immunosuppressor cells which might be induced by the immunization, serum or cell transfer experiments were performed. In other words, whether the enhancement was abrogated by transfer of serum or by transfer of thymus or spleen cells from the rats treated with immunization alone was considered. In the serum transfer experiment, 1 ml of serum obtained from rats 6, 8, and 10 days after immunization with X-irradiated KMT-17 cells was transferred i.v. on Days 6, 8, and 10, respectively, into rats immunized on Day 0 and given BU on Day 5. KMT-17 cells (1 × 10⁵) were then injected s.c. on Day 10. As shown in Table 6, transfer of serum did not affect the enhancement of antitumor transplantation resistance by BU. In the cell transfer experiment, 400 million thymus cells or 2 × 10⁶ spleen cells were adoptively transferred i.v. on Day 9 into WKA rats immunized with X-irradiated KMT-17 cells on Day 0 and then given BU on Day 5. As shown in Table 7, enhanced antitumor transplantation resistance by BU was significantly abrogated by transfer with thymus cells and was slightly abrogated with spleen cells obtained from rats 9 days after immunization with X-irradiated KMT-17 cells alone. Transfer with thymus or spleen cells from normal, immunized plus BU-treated, and WFT-2N-immune rats did not affect enhancement by BU. These results suggest that the enhancement of antitumor transplantation resistance by BU was due to the

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5 X. Kodama, unpublished data.
selective elimination of tumor-specific immunosuppressor cells from the immunized hosts, although the possibility of humoral factor(s) cannot be completely excluded due to the limits of our experiments.

DISCUSSION

Many reports on the immunosuppressive effects of anticancer drugs have been published (1, 16). Reports on their immunostimulatory effects have also been published but are fewer in number. While cyclophosphamide, 6-mercaptopurine, vincristine, and a few other drugs have been reported to be anticancer drugs showing immunostimulatory effects (2, 4–6, 8, 9, 14, 17, 19, 21, 22, 26), most of their immunostimulatory effects have been observed in antibody production or in delayed hypersensitivity. Few reports have been found on immunostimulation by anticancer drugs when given either before or after immunization.
The precise mechanisms of enhancement of antitumor transplantation resistance by BU are still unclear. However, from the results of cell transfer experiments, in which the enhancing effects of BU were significantly abrogated by adoptive transfer with thymus cells and were slightly abrogated with spleen cells from rats receiving only immunization with X-irradiated tumor cells, we speculate that BU inhibited the immunosuppressor cells more selectively than it did killer cells; as a result, the activities of the killer T-cells were enhanced. Immunosuppressor cells have been demonstrated in the thymuses and spleens of tumor-bearing animals by Umiei and Trainin (23) and Fujimoto et al. (3). In our studies, immunosuppressor cells were also demonstrated in the thymuses of the immunized rats. The spleens contained 2 subpopulations of lymphocytes, killer cells and immunosuppressor cells, which were detected by the Winn assay and by the adoptive transfer system, respectively. Similar observations have been reported by Small and Trainin (18) and by Kimura and Aoki (11). Assuming that antitumor resistance is the sum of killer cell and suppressor cell activity, the following possibilities seem reasonable. (a) Elimination of the suppressor cells would result in the enhancement of antitumor resistance. (b) BU may inhibit the production of humoral blocking factors, which inhibit cell-mediated immunity (5, 6, 12). Since serum transfer did not affect the enhancing effects of BU, this possibility seems unlikely, although it cannot be completely excluded due to the limits of our experiments. (c) BU may enhance the macrophage tumoricidal function (15, 20). However, from the results of carrageenan treatment and of anti-T-serum treatment of spleen cells, this possibility seems unlikely. (d) The BU-induced rebound hyperplasia of lymphoid organs may enhance immunity. However, the lymphoid organs of WKA rats treated with BU were rather atrophic, suggesting that this possibility could not be responsible for our experimental results. Further experiments are required with regard to other possibilities, such as the theory that BU may induce production of interferon or that nucleic acid released from cells injured by BU stimulate the immune response to tumor cells (2).

In preliminary experiments, similar enhancement was also observed when cyclophosphamide, 40 mg/kg, was given 5 days before immunization with X-irradiated KMT-17 tumor cells and florafur, 300 mg/kg, and mitomycin C, 1 mg/kg, were given 5 days after immunization, whereas Adriamycin, 4 mg/kg, showed no enhancing effects either before or after immunization. On the other hand, cyclophosphamide and mitomycin C were rather immunosuppressive when given after and before immunization, respectively. These results indicate that some other anticancer drugs besides BU exhibit time-dependent enhancing or suppressing effects on antitumor transplantation resistance, although we think that dose and time parameters are necessary before conclusions can be reached. The reasons for the difference in effective timings for enhancement of antitumor resistance for each drug are still unknown. However, if we consider the above reasons, in a comparison of BU with cyclophosphamide, it can be speculated that cyclophosphamide inhibits immunosuppressor cells selectively when given before immunization and that it inhibits immunosuppressor cells as well as killer cells when given after immunization whereas BU inhibits immunosuppressor cells selectively when given either before or after immunization. Differences in effects on the immunocompetent cells may be related to the differences in the effective timing of the 2 drugs. Further investigations are required as to the mechanisms of effective timing for enhancement by florafur and mitomycin C.

REFERENCES


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