Failure of Bleomycin to Affect Humoral or Cell-mediated Immunity in the Mouse

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SUMMARY

Swiss CD-1 and C57BL/6 X DBA/2 F₁ mice were treated with a variety of dosage schedules of bleomycin. Antigenic challenge with sheep erythrocytes or leukemia L1210 was used to test humoral or cell-mediated immunity, respectively. Bleomycin had no effect upon serum hemagglutinin titers, including mercaptoethanol-resistant antibody, or upon hemagglutinin antibody-producing cells in the spleen, as assayed by immunocytoadherence. Spleen cell-mediated immunity against leukemia L1210, measured by a quantitative in vitro assay, was unaffected except at toxic (lethal) doses. We conclude that this new cancer chemotherapeutic agent lacks immunosuppressive activity in the mouse.

INTRODUCTION

Recently, bleomycin, a new antineoplastic agent with several desirable characteristics, was introduced. This water-soluble, basic, glycopeptide antibiotic complex has been observed to have a distinct antitumor activity, particularly against squamous cell carcinomas, in both animals and man (7–11, 17, 18, 20–23). The drug has been postulated to bind to DNA, causing a single-strand scission in the presence of a sulphydryl compound, thereby inhibiting DNA synthesis (16).

Two of the major problems associated with the use of cancer chemotherapeutic agents have been the concomitant production of pancytopenia and immunosuppression (10, 15). Of particular interest therefore has been the apparent lack of hematopoietic depression associated with the clinical use of bleomycin. However, only a few laboratory investigations have dealt specifically with the effect of bleomycin on the immune response. Ohno et al. (14) have demonstrated an apparent lack of immunosuppressive effect of bleomycin on antibody synthesis, even at high dose schedules, although Mathé (11) has cited unpublished evidence of immunosuppressive activity on hemolysin-forming cells. More recently, Andrews (1) also reported a lack of suppression by bleomycin on cellular immunity except at high dosage levels. However, the skin allograft system that he utilized is a complex one in which the specific effects of a drug upon lymphocytes are difficult to quantitate. Therefore, the following studies were undertaken to provide more quantitative data on the effect of bleomycin on both humoral and cell-mediated immunity at a variety of schedules. The results indicate that bleomycin has no effect on humoral immunity and has an immunosuppressive effect on cell-mediated immunity only at toxic dose schedules.

MATERIALS AND METHODS

Bleomycin

Bleomycin (Blenoxane) was obtained in vials containing 15 mg of the drug from Bristol Laboratories, Syracuse, N. Y. The antibiotic was dissolved in 0.9% NaCl solution and administered to mice i.p. in a volume of 0.05 to 0.2 ml in various dosage schedules.

Humoral Immunity

Mice. Swiss CD-1 females (Charles River Laboratories, Wilmington, Mass.) and C57BL/6J X DBA/2J F₁ (hereafter called B6D2F₁) males (The Jackson Laboratory, Bar Harbor, Maine) 10 to 14 weeks old, were the recipients of antigen in experiments concerned with humoral antibody synthesis. Five to 10 mice constituted each group. Since the results were similar for both sets of mice, they have been combined for presentation.

Immunization with SRBC and Titration of Immunity.

One-tenth ml of a 10% suspension of SRBC (approximately 1.7 X 10⁸ cells) washed 3 times with 0.9% NaCl solution was injected i.p. into all experimental groups as the antigen. 0.9% NaCl solution (0.1 ml i.p.) was administered to controls. In 1 experiment, 0.4 ml blood was collected after warming the animals by nicking the tail vein of all 10 mice in each group on Days 3, 7, 10, and 14. In other experiments, mice were exsanguinated via the jugular vein (1 ml) in groups of 5 or 6 on Day 7 after administration of antigen. Hemagglutinins were titrated in the serum by microassay (6). Titer was expressed as the log₂ of the reciprocal of the last dilution of serum causing macroscopic agglutination.

The spleen of each mouse was also removed and assayed for hemagglutinin-forming cells by the immunocytoadherence (rosette) technique of Biozzi et al. (2). This technique, although giving lower estimates of antibody-producing cells than the hemolytic plaque-forming cells assay, is the appropriate cellular counterpart of the class of antibodies measured in

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2Leukemia Society Scholar. To whom requests for reprints should be addressed at Yale University School of Medicine, 333 Cedar Street, New Haven, Conn. 06510.
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the serum. We have successfully used these methods to discriminate among drug treatment groups previously (6).

**Cell-mediated Immunity**

Mice. CD-1 females were the recipients of the antigen, leukemia L1210 cells, in all experiments dealing with cellular immunity. The animals varied in age from 10 to 14 weeks, but in a given experiment all mice were of the same age. DBA/2J mice (The Jackson Laboratory) were used to maintain leukemia L1210 in its ascites form, which was transferred weekly.

**Immunization with Killed Leukemia L1210 Cells.** The antigen given i.p. in all the experiments dealing with cell-mediated immunity consisted of $5 \times 10^7$ mitomycin C-killed leukemia L1210 cells. This number of killed cells was found to lead consistently to high levels of cell-mediated immunity in these mice (12). Since the cells were killed, any effect that bleomycin might have had upon the leukemia was eliminated, allowing a constant amount of antigen to be administered to each group. Mitomycin C was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Leukemia L1210 cells were incubated with mitomycin C, 25 $\mu$g/10$^6$ tumor cells, for 1 hr at 37°. Suspensions were then washed 3 times in Fischer's medium for 10 min at 1000 rpm in order to remove the drug. The final cell suspensions were pooled, and the volume was adjusted to give a concentration of $5 \times 10^7$ cells/0.2 ml. Injection of $5 \times 10^7$ presumably dead cells into normal allogeneic cells, we have frequently noted 10 to 15% fewer mastocytes in their presence compared with tumor cells in medium alone. Thus lytic effects are somewhat understated by this calculation but are more clearly attributable to the immunization rather than the allogeneic nature of the effector lymphoid cells. Student's $t$ test was used to evaluate statistical significance.

**RESULTS**

**Humoral Immunity**

**Effects of Bleomycin on Hemagglutinin Titers and Agglutinin-forming Cells in the Spleen.** A variety of bleomycin schedules were tested for their effect (Chart 1; Table 1). Chart 1 shows that bleomycin failed to affect the hemagglutinin titer of mice at a dose of 25 mg/kg/day for 5 days. Days 0 to 4 after administration of antigen. Peak titers were reached 7 days after antigen in experimental mice, and in control mice treated with 0.9% NaCl solution. No delayed decrease in titer occurred in bleomycin-treated mice thereafter. Mice treated with an immunosuppressive dose of cytarabine are also depicted for comparison, showing a delayed rise in titer evident at Day 7. To facilitate comparison of several schedules, only titers at Day 7 were measured in other experiments, since even temporary immunosuppression is detectable at that time. None of the other schedules had any consistent effect upon the formation of hemagglutinins, either as measured as a serum titer or as the proportion of hemagglutinin-forming cells in the spleen. Even at doses that led to weight loss or mortality of a proportion of the group, such as 200 or 300 mg/kg 2 days

![Chart 1. Hemagglutination titers of mice treated with 0.9% NaCl solution, Bleomycin, 25 mg/kg/day, or cytarabine, 40 mg/kg/day. Days 0 to 4 after 0.1 ml of 10% SRBC. Blood from pairs of mice was pooled before assay. Each point, results with 10 mice (5 pairs) studied serially. Note the lack of effect of bleomycin, compared with cytarabine, which led to a delay in appearance of peak titers. Even temporary immunosuppression is still evident on Day 7 with this dosage.](chart)
Table 1

<table>
<thead>
<tr>
<th>Dose of bleomycin (mg/kg)</th>
<th>Administration on Day</th>
<th>Mean hemagglutinin-forming cells/10^3 spleen cells</th>
<th>Mean hemagglutination titer</th>
<th>Toxicity (no. dead)</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>4.8</td>
<td>4.8</td>
<td></td>
<td></td>
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<tr>
<td>62.5</td>
<td>-2</td>
<td>3.9</td>
<td>5.0</td>
<td></td>
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<td>5.4</td>
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<td>-2</td>
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</tr>
<tr>
<td>300</td>
<td>-2</td>
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<td>3.5</td>
<td>1</td>
</tr>
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<td>12.5</td>
<td>0</td>
<td>2.3</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
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<td>0</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.3</td>
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<td>0–4</td>
<td>3.3</td>
<td>5.0</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results from 3 spleens were averaged for each value, with 12 values averaged for controls. The background of rosettes in an untreated mouse was 0.7/10^3 cells.

<sup>b</sup> Expressed as log<sub>2</sub> of the reciprocal of the end point dilution of serum, measured on Day 7. Serum titer was 0 (<2) in the same normal mice.

<sup>c</sup> ND, not done.

Table 2

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Dose (mg/kg)</th>
<th>Days of administration</th>
<th>% lysis</th>
<th>No. of subjects&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
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<td>A. Course of 5 daily doses</td>
<td>12.5</td>
<td>0–4</td>
<td>51.65 ± 1.52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0–4</td>
<td>52.13 ± 1.07</td>
<td>9</td>
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<td>35</td>
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<td>36.47 ± 1.91</td>
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</tr>
<tr>
<td></td>
<td>50</td>
<td>0–4</td>
<td>22.22 ± 1.28</td>
<td>9</td>
</tr>
<tr>
<td>B. Single doses</td>
<td>125</td>
<td>2</td>
<td>50.09 ± 0.29</td>
<td>4</td>
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<td></td>
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<td>47.42 ± 2.06</td>
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</tr>
<tr>
<td>C. Control</td>
<td>None</td>
<td>51.15 ± 1.10</td>
<td>18</td>
<td></td>
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</table>

<sup>a</sup> All assays were performed on spleens from individual mice. Two groups of 4 or 5 experimental mice were compared with controls in the same experiment. Two assays are summarized in Experiment A, with similar assay results in both, as indicated by the S.E.

<sup>b</sup> Mean ± S.E.

before antigen treatment, no immunoosuppressive effect was demonstrable. Similarly, the mercaptoethanol-resistant, presumably IgG antibody (IgG) titer of all sera on Day 7 was approximately 2 (i.e., 1:4 serum dilution), regardless of whether an 0.9% NaCl solution placebo or the active drug was administered.

Cell-mediated Immunity

Treatment with a Course of Bleomycin after Antigen Administration. Mice received daily i.p. injections of either 12.5, 25, 35, or 50 mg of bleomycin per kg of body weight from Day 0 to Day 4. Control animals were sensitized with L1210 cells and received corresponding daily injections of 0.9% NaCl solution. The results (Table 2) show that bleomycin given daily in doses of 12.5 or 25 mg/kg following the administration of antigen had no effect on cellular immunity, as compared to controls. Spleen cells from mice receiving 35 or 50 mg of bleomycin per kg daily showed a 14.7 and 28.9% decrease, respectively, from immune control values in their ability to lyse target cells. The higher degree of immunosuppression at 50 mg/kg was significant (p < 0.001), compared with that of mice receiving 35 mg/kg. However, a daily dose of 35 mg/kg, resulted in a 15% weight loss and severe debility in all mice so treated, as well as the death of 2 of 11. A daily dose of 50 mg/kg resulted in a 25% weight loss and the death of 9 of 18.

Single doses of either 125 or 200 mg of bleomycin per kg of body weight (roughly comparable to 5-day treatments of 25 or 35 mg/kg/day) were administered to mice 2 days after the antigen. No effect on cellular immunity was noted (Table 2).
DISCUSSION

A major drawback of most cancer chemotherapeutic agents has been their lack of a preferential effect on the tumor cells, affecting instead, all rapidly growing cells. Most agents studied have caused immunosuppression, presumably by inhibiting proliferating lymphocytes (10). However, 2 drugs, mithramycin (M. S. Mitchell, unpublished data), which has been used to combat embryonal carcinoma of the testis, and the antimelanoma agent 5-(3,3-dimethyl-1-triazeno (DTIC) (3, 5, 13), have been found to lack immunosuppressive activity in humans.

Ohno et al. (14) reported that single doses of 50 mg/kg (10% lethal dose) or 75 mg/kg (20% lethal dose) 2 days after antigen did not suppress the formation of circulating antibody in C57BL/6 mice immunized with SRBC. They reported that hemolytic plaque-forming cells in spleens of these mice were minimally suppressed by the above-mentioned doses of bleomycin, but the degree of suppression was insignificant biologically, compared with the effect of daunorubicin at a dose of 19 mg/kg (10% lethal dose). Andrews (1) recently evaluated the immunosuppressive action of bleomycin in an allograft system. He found that at high doses (e.g., 105 mg/kg in 4 divided daily doses), the drug significantly altered graft rejection times. However, the high mortality and severe debility at such a schedule suggested that the prolonged allograft survival times were attributable to the animals’ poor general condition rather than to specific immunosuppression. Our own results clearly indicate that bleomycin has no effect on humoral immunity, even at toxic doses, and has suppressive activity on cellular immunity only at toxic doses.

Except for a report of inhibition of the response of human lymphocytes to mitogens in vitro (19), no data are yet available on the effect of bleomycin in man. If our findings were found to apply also to humans, they would suggest that bleomycin does not impair the cellular immune response to tumor-associated antigens that is critical in the rejection process. The absence of immunosuppression, like failure to suppress hematopoietic elements, would be another highly desirable characteristic of this antitumor agent.

REFERENCES

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