Marked Immunosuppression With Minimal Myelosuppression by Bleomycin In Vitro

By Glenn Tisman, Victor Herbert, Le Teng Go, and Lois Brenner

Bleomycin, in concentrations similar to those present after drug administration, was a potent inhibitor in vitro of both human phytohemagglutinin (PHA)-stimulated and pokeweed mitogen (PMW)-stimulated lymphocyte transformation, but lower concentrations were much more inhibitory to the former. Bleomycin showed minimal or no in vitro myelosuppression. The similarity of these in vitro results to in vivo results by others suggests the in vitro system used here may be useful to screen new drugs for potential in vivo suppression of either the myeloid or the lymphoid tissue. The data suggest that by adjusting the dose of bleomycin inhibition of lymphocytes stimulated by PHA with only minimal inhibition of lymphocytes stimulated by PWM may be possible. Variable response of lymphoid neoplasia to bleomycin may in part reflect whether the cell type is T or B in origin, in view of work by others suggesting that PHA is more stimulatory to T cells and PWM is stimulatory to both B and T cells.

BLEOMYCIN is a mixture of glycopeptides isolated from a strain of Streptomyces verticillus. The drug has been shown to have antitumor activity with minimal, if any, in vivo myelosuppression. Japanese workers reported that bleomycin lacks immunosuppressive effects on the primary response of mice to sheep red blood cells but inhibited in vitro phytohemagglutinin (PHA)-induced blastogenesis of human peripheral lymphocytes. The current study (a preliminary report of which appeared in abstract form) confirms that bleomycin inhibits PHA-induced blastogenesis of human lymphocytes, reports dose-dependent inhibition of pokeweed mitogen-induced blastogenesis, and demonstrates minimal to no in vitro myelosuppressive activity against human bone marrow cells. The in vitro system used in the present study appears useful in predicting in vivo action of new drugs on the immune and myeloid systems.
MATERIALS AND METHODS

Blood and bone marrow specimens were obtained in the course of requested hematologic evaluation and with informed consent from patients on the general medical and hematology wards. The patients included individuals with diabetes mellitus, alcoholism, infection, or neoplasm, plus normal volunteers. None was receiving myelosuppressive therapy.

Lymphocyte Collection and Culture

Heparinized blood specimens (30 U heparin/ml) were allowed to sediment in the original syringe, placed nozzle-up at a 45° angle at room temperature for 2 hr. The leukocyte-rich plasma was then expressed through a Butterfly-19 infusion tube (Butterfly-19 Infusion Set, No. 4590, Abbott Labs., North Chicago, Ill.), by pressing the plunger of the syringe, into a 50 ml sterile conical test tube. The cells were separated by centrifugation at 500 g for 10 min and were washed once in 10 ml of McCoy's 5A medium (Microbiological Associates, Bethesda, Md.). The yield of lymphocytes was between 50% and 80%, with a lymphocyte purity of 50%-90% and 95% viability as measured by Trypan blue dye exclusion. The washed cells were then cultured in 16 X 125 mm Falcon (Falcon Plastics, Oxnard, Calif.) plastic tubes (0.5 X 10^8 cells/ml) in 3-ml aliquots of McCoy's 5A medium to which had been added 20% fetal calf serum (Flow Labs., Rockville, Md.). Difco phytohemagglutinin P (PHA-P) (Difco Labs., Detroit, Mich.) was reconstituted with 5 ml of McCoy's 5A medium, and pokeweed mitogen (PWM) (Gran Island Biologicals, Grand Island, N.Y.) was reconstituted with 10 ml of McCoy's 5A medium; 100 µl of PHA-P and 50 µl of PWM were added to each tube except for mitogen controls. Bleomycin (Bristol Labs., Syracuse, N.Y.), in varying concentrations, was added to cultures and was allowed to incubate at 37°C for 3 days in a 5% CO₂ atmosphere, after which 0.1 ml of 125I-labeled UdR, 10 µCi/ml (specific activity = 2 mCi/mg; Amersham/Searle) was added, and incubation was continued for another 4 hr. The cells were then transferred to glass tubes, centrifuged at 500 g for 10 min, and the supernate was discarded. The cell pellet was washed twice with 3 ml of Hank's Balanced Salt Solution (HBSS). After washing the cells, one drop of 25% salt-poor human albumin was added to each culture, and then 3 ml of cold 10% TCA were added. The supernate, after centrifugation at 800 g for 10 min, was discarded and washed once more with 3 ml of 10% TCA. The precipitate was then counted in a Picker Autowell II γ counter.

Morphologic evidence of lymphocyte transformation was obtained by microscopic examination. It was found to be an inadequate quantitative index of transformation because of the presence of cell clumps and intermediate transformed cells. Trypan blue studies after PHA-P were difficult to interpret due to clumping. However, there was no apparent difference between control cells and cells cultured in the presence of bleomycin.

Short-term Bone Marrow Culture

The techniques employed were essentially those previously used in this laboratory. Ten to 20 ml of bone marrow was aspirated from the posterior superior iliac spine into a syringe containing Tris-Hank's buffered salt solution plus 200 U/ml each of penicillin and streptomycin (THBSS) and 100 mg of heparin. The cells were passed through a 50-mesh, 0.003 inch gauge wire screen and washed with a 10 ml aliquot of THBSS. The cells were then suspended in autologous serum-enriched (25%) THBSS, and 0.3 ml aliquots were added to 10 ml B-D Vacutainer tubes (Becton-Dickinson, Inc., Rutherford, N.J.). Cell count ranged between 2 and 12 X 10^6/ml of final culture. One-tenth milliliter aliquots of a stock solution of bleomycin (170 µg/ml) of 5-fluorouracil (250 µg/ml) were added, and the final volume was adjusted to 0.9 ml with THBSS. After 1-hr incubation in a Dubnoff metabolic shaker-incubator at 37°C, 0.1 ml of ³HTdR (10 µCi/ml, specific activity = 25 Ci/mM) was added and incubation continued for 3 hr more. After incubation, the cells were washed in THBSS and were then shock lysed to remove hemoglobin. TCA at 4°C was added as in lymphocyte cultures, and the radioactivity of the acid insoluble material was determined as an index of incorporation of ³HTdR into DNA.
RESULTS

As can be seen in Fig. 1, bleomycin, in concentrations between 0.1 and 200 \( \mu g/ml \) of culture (maximum blood levels achieved in humans after giving 15 mg intravenously = 3.3 \( \mu g/ml \)) was effective in inhibiting PHA-P-induced lymphocyte transformation to a much greater degree than PWM induced transformation. Such inhibition was always greater for the PHA-P response, whether the PHA-P maximum response was greater than, less than, or equal to the PWM maximum response.

Concentrations of bleomycin equal to 17.5 \( \mu g/ml \) of culture showed only slight to moderate inhibition of \(^{3}\)HTdR incorporation into human bone marrow DNA (Table 1). In one instance, a dose-response curve was plotted with varying bleomycin concentrations vs. both transforming (PHA) lymphocytes and bone marrow cells from one patient grown in short-term culture (Fig. 2). Incubation with 5-fluorouracil showed the expected inhibition of the de novo (i.e., from deoxyuridine) pathway to thymidine synthesis in both lymphocyte and bone marrow cultures, with resultant increase in \(^{3}\)HTdR incorporation via the salvage (i.e., from thymidine) pathway representing toxicity to DNA synthesis and suggesting that the bleomycin effect, whatever it may be, is not dominantly on the de novo pathway.
Table 1. Effects of Bleomycin and 5-Fluorouracil (5-FU) on Incorporation of ³HtdR Into Human Bone Marrow DNA

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Bleomycin (17.5 μg/ml)</th>
<th>5-FU (25 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95 ± 8†</td>
<td>154 ± 12</td>
</tr>
<tr>
<td>2</td>
<td>78 ± 6</td>
<td>156 ± 6</td>
</tr>
<tr>
<td>3</td>
<td>86 ± 12</td>
<td>217 ± 13</td>
</tr>
<tr>
<td>4</td>
<td>102 ± 6</td>
<td>187 ± 15</td>
</tr>
<tr>
<td>5</td>
<td>76 ± 4</td>
<td>227 ± 18</td>
</tr>
<tr>
<td>6</td>
<td>95 ± 9</td>
<td>175 ± 9</td>
</tr>
<tr>
<td>7</td>
<td>75 ± 8</td>
<td>168 ± 15</td>
</tr>
</tbody>
</table>

*In all cases, ³HtdR incorporation into DNA was measured as cpm, and cpm was always at least 100 times greater than background. Radioautographs of marrow cells after incubation revealed grains over both red and white blood cell precursor nuclei.
†Mean ± 1 standard deviation. All numbers are from triplicate determinations of percentages of control value and are calculated from the formula:

Radioactivity in DNA of marrow cells incubated with ³HtdR in presence of bleomycin × 100

Radioactivity in DNA of cells incubated with ³HtdR in absence of bleomycin

**DISCUSSION**

Ohno et al.³ noted lack of in vivo immunosuppression by bleomycin in mice, as measured by plaque formation by spleen cells and by humoral antibody response to sheep red blood cells. They did, however, report inhibition by bleomycin of PHA-P-induced lymphocyte transformation in vitro. Their conclusion was that bleomycin was not immunosuppressive in...
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vivo. Because spleen cell plaque formation and humoral antibody production are measurements primarily of B lymphocyte activity, although T cells may facilitate B cell function,\textsuperscript{9,10} we considered the possibility that bleomycin might preferentially inhibit T cell transformation.

Response to PWM is a measure of both B and T lymphocyte function, but response to PHA-P is mostly a T cell function.\textsuperscript{11-16} Much lower concentrations of bleomycin inhibited PHA-P-induced more than the PWM-induced transformation response. This may be one of many possible explanations for failure of Ohno et al. to demonstrate inhibition by bleomycin of B cell function in the mouse.

The minimal suppression of \textsuperscript{3}H TdR incorporation into DNA of human bone marrow cultures fits the clinical observations of minimal, if any, bone marrow toxicity induced by concentrations of bleomycin extremely toxic to lymphocytes.\textsuperscript{1,2}

If bleomycin is as effective in vivo as suggested by this in vitro data, then it would be a particularly useful drug for immunosuppression, manifesting only minimal if any bone marrow toxicity. Such appears to be the case in studies so far carried out in patients with lymphomatous diseases,\textsuperscript{1} in which marked (malignant) lymphocyte suppression was accompanied by minimal myelosuppression.

Selective inhibition by bleomycin of PHA-P-responsive cells, at concentrations of drug lower than those present when used in chemotherapy, may allow its use as an immunosuppressive agent at very low concentrations. The greater sensitivity of PHA-P-responsive lymphocytes to bleomycin may relate to variable responses of lymphomas to the drug. Lymphoma response could be related to whether it was primarily derived from PHA-P- or PWM-responsive cells.

REFERENCES

10. Rubin AS, Coons AH: Specific heterologous enhancement of immune response:
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