

The Effects of Bleomycin on Immunocompetence in Man¹

Daniel E. Lehane, Elizabeth Hurd, and Montague Lane

The Sadie Darsky Laboratory for Clinical Cancer Research, Departments of Pharmacology [D. E. L., E. H., M. L.] and Medicine [D. E. L., M. L.], Baylor College of Medicine, Houston, Texas 77025

SUMMARY

Bleomycin was administered to six patients with advanced cancer. Multiple parameters of both antibody- and cell-mediated immunity were followed serially to characterize the effects of bleomycin on immunocompetence in humans. Antibody-mediated immunity, including primary vaccination with keyhole limpet hemocyanin, was not depressed. While there was no significant suppression of cell-mediated immunity, phytohemagglutinin-stimulated lymphocyte blastogenesis was reduced after treatment with bleomycin.

The *in vitro* effects of bleomycin on lymphocyte stimulation were studied, and while thymidine incorporation was significantly inhibited by bleomycin, leucine incorporation was not reduced even at high concentrations of bleomycin. We have concluded that bleomycin does not suppress immunocompetence in man.

INTRODUCTION

Most chemotherapeutic agents have been found to suppress immune function (8). Recovery of immunological function following chemotherapy has been correlated with a better response to therapy (4). Bleomycin is an antitumor antibiotic effective against several types of cancer, including lymphomas (2). Its major toxic effects are mucositis, dermatitis, pulmonary fibrosis, and minimal myelosuppression (2). Several investigators have reported that bleomycin is not immunosuppressing in animals (1, 5, 16, 21). In the present studies, the effects of bleomycin on immunocompetence have been evaluated in human subjects. The drug was shown not to impair humoral or cellular immunity.

MATERIALS AND METHODS

Subjects. Immunocompetence was measured in a group of 6 patients with advanced disseminated cancer who were being treated i.m. with bleomycin (Blenoxane) (Bristol Laboratories, Syracuse, N. Y.), 15 mg/sq m, twice weekly (3 squamous cell carcinomas of the lung, and 1 each of adenocarcinoma of the colon, adenocarcinoma of unknown primary site, and squamous cell carcinoma of the esophagus). Prior to each dose of bleomycin, the patients were examined for drug toxicity and response to therapy. If

toxicity was evident, treatment was delayed until it resolved completely. Informed consent was obtained from each patient prior to immunological testing.

Immunological Testing. Patients were tested serially 2 days prior to starting treatment, and after 2 and 4 weeks of bleomycin therapy. Six normal volunteers were selected from the ward and laboratory personnel to serve as controls for immunological testing.

Humoral Immunity. Immunoglobulins and serum complement levels were measured by the radial immunodiffusion technique. Isoantibodies to human blood group antigens were measured by the saline agglutination technique as modified in this laboratory for the microtiter system (11). Recall virus antibody titers to poliovirus type 1 and herpes simplex virus type 1 were measured by the microneutralization test. Cytomegalovirus antibody was measured by the microtiter complement fixation test.

Primary vaccination with KLH² (Calbiochem, San Diego, Calif.) was performed by administering 1 mg intradermally 2 days prior to beginning bleomycin treatment. KLH antibody was measured by the passive hemagglutination test adapted to the microtiter method as described previously (11). Anti-KLH IgM antibody titers were determined by the 2-mercaptoethanol technique.

Cellular Immunity. Recall delayed hypersensitivity reactions were measured after intradermal injection of each of the 6 skin test antigens: mumps (0.1 ml, Lilly, Indianapolis, Ind.), Varidase (0.1 ml containing 100 units of streptokinase; Lederle, Pearl River, N. Y.), purified protein derivative (Aplisol, 5 tuberculin units/0.1 ml; Parke Davis & Co., Detroit, Mich.), Monilia mixture, Dermatophytin, and Dermatophytin "O" (0.1 ml of a 1:10 dilution; Hollister-Stier, Spokane, Wash.). Primary delayed hypersensitivity reaction to KLH was measured by injecting 1 mg in 0.1 ml 0.9% NaCl solution intradermally. All skin tests were read at 48 hr, and the mean diameter of induration measured in 2 perpendicular diameters was recorded. On the basis of these measurements, the average diameter of all skin tests with measurable induration was calculated for each patient.

Lymphocyte blastogenesis following PHA stimulation was performed with a microtiter culture system and [³H]thymidine incorporation as an indicator of blastogenesis. Lymphocytes prepared by the Ficoll-Hypaque technique were suspended in Roswell Park Memorial Institute Medium 1640 (4 × 10⁶/ml; Grand Island Biological Co., Grand Island, N. Y.) supplemented with 20% fetal calf

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² The abbreviations used are: KLH, keyhole limpet hemocyanin; PHA, phytohemagglutinin.

serum (Grand Island Biological Co., Grand Island, N. Y.). Each culture well contained 50 μ l of the lymphocyte suspension, 50 μ l of PHA-M (Grand Island Biological Co., Grand Island, N. Y.) diluted 1:10 in Roswell Park Memorial Institute Medium 1640, and 100 μ l of growth medium. Replicate cultures were incubated at 37° for 72 hr. Tritiated thymidine was added 3 hr prior to harvesting for liquid scintillation counting, which was performed with a multisample precipitator (Otto Hiller Co., Madison, Wis.). Results are expressed as a stimulation index, which is the ratio of the mean cpm in PHA-stimulated cultures to those of unstimulated control cultures.

Lymphocyte sheep erythrocyte rosettes were prepared by mixing equal volumes of lymphocytes (1×10^7 /ml) suspended in phosphate-buffered saline (pH 7.2) with 10% fetal calf serum added, and the sheep red blood cells (0.5%); this mixture was then centrifuged to a pellet at $200 \times g$ for 5 min, incubated at 4° for 30 min, gently resuspended and counted in a hemocytometer (20). Rosettes are recorded as the percentage of lymphocytes counted.

The *in vitro* effect of bleomycin on PHA stimulation of normal human lymphocytes, utilizing the thymidine incorporation assay, was measured in 7 normal volunteers. Bleomycin was added to the culture medium at final concentrations of 0.0015, 0.015, and 0.15 mg/ml. The average plasma concentration of bleomycin achieved in man is approximately 0.0015 mg/ml, and the average tissue concentration of bleomycin in sensitive tumors has been reported to be as high as 0.15 mg/ml (6). After 1 hr of incubation, one-half of the lymphocyte cultures containing bleomycin were centrifuged, and the lymphocytes were washed with growth medium to remove bleomycin, thus simulating the *in vivo* clearance of bleomycin.

Lymphocytes from 6 volunteers were tested by the method described by Levy and Kaplan (13), in which serial dilutions of PHA were cultured with lymphocytes for 20 hr in leucine-free media to which [3 H]leucine, 0.5 μ Ci/well, was added. Cultures containing bleomycin for the duration of the culture period, cultures from which the bleomycin was removed after 1 hr, and cultures without bleomycin were performed simultaneously in both the [3 H]thymidine-incorporation and the [3 H]leucine-incorporation experiments.

Statistical Methods. Results from patients prior to chemotherapy were compared with the results obtained at both 2 and 4 weeks during bleomycin treatment. The significance of the differences between means were evaluated by the analysis of variance and by Student's *t* test.

RESULTS

Clinical Results. Bleomycin toxicity developed in 2 patients. Both experienced moderate stomatitis, and 1 developed moderately symptomatic pulmonary fibrosis several weeks after bleomycin treatment had been discontinued. This resulted in the omission of 1 dose of bleomycin for 1 patient and 2 doses for the other. There was no myelosuppression. One patient with squamous cell carcinoma of the esophagus experienced a subjective antitumor response.

Humoral Immunity. The results of humoral immunity testing are shown in Table 1. Established humoral immunity was evaluated by measuring immunoglobulin levels, isoantibody titers, and virus antibody titers. There were no significant changes in these parameters when the pretreatment values were compared with the values obtained during bleomycin therapy. Isoantibody titers were significantly lower than controls, but there was no change in isoantibody titers during bleomycin treatment. Poliovirus, herpes simplex virus, and cytomegalovirus antibody titers were also unchanged throughout the treatment period, although herpes simplex antibody titers were significantly elevated in patients.

The patients were able to make a normal primary immune response to KLH while receiving bleomycin. Antibody titers were very low 2 weeks postvaccination, comparable to controls. Titers had increased by 4 weeks, and patients had begun to make IgG antibody. At 4 weeks, 54% of the measurable antibody titer was 2-mercaptoethanol sensitive in the patient group and 64% in the controls.

Cellular Immunity. The results of cellular immunity testing are shown in Table 2. While the mean diameter of positive skin tests was significantly less than that of normal controls, there was no change in recall delayed hypersensitivity reactions in patients treated with bleomycin. Primary delayed hypersensitivity testing with KLH showed values less than controls, which decreased during the treatment period, but the difference was not statistically significant. Lymphocyte blastogenesis after PHA stimulation was normal prior to bleomycin therapy, fell markedly after 2 weeks, and increased by the 4th week of treatment. The fall in PHA stimulation index was not significant ($p = 0.2$) at 2 weeks.

In Vitro Studies. The effects of bleomycin *in vitro* on lymphocyte blastogenesis are shown in Table 3 and Chart 1. When PHA-stimulated lymphocytes were cultured in the presence of bleomycin for the entire culture period, there was significantly reduced thymidine incorporation which was dose related (Table 3). If bleomycin was removed from the cultures after 1 hr of incubation, thymidine incorporation was reduced to levels less than those of controls at high doses of bleomycin, but not to levels significantly different from those of controls.

Lymphocyte stimulation was also measured by the [3 H]leucine-incorporation technique (Chart 1). It is evident, using this assay for blastogenesis, that bleomycin had no effect on PHA lymphocyte stimulation even when high concentrations of bleomycin were maintained in the culture media for the entire duration of the experiment.

DISCUSSION

With the exception of a few drugs, *i.e.*, imidazole carboxamide and mithramycin, cancer therapeutic agents have been found to be immunosuppressants (3, 8). However, it has been shown by several investigators (4, 7, 12) that proper drug administration scheduling permits rapid rebound from immunosuppression, and overshoot has been observed. Recovery of immunocompetence has been associated with a good response to chemotherapy. Bleomycin has been associated with little myelosuppression in humans and

Table 1
Effects of bleomycin on antibody-mediated immunity

The mean scores for 6 patients who were treated with bleomycin and tested immunologically both prior to therapy and after 2 and 4 weeks of bleomycin treatment are compared with normal control scores.

	Serum immunoglobulins ^a						Recall virus antibody titers ^b				KLH antibody titers ^b	
	IgG	IgM	IgA	Complement	Polio	Herpes simplex	Cytomegalo-virus	Isoantibody titers ^b	Patients	Controls		
Prestudy	1438 ± 154	154 ± 60	305 ± 34	138 ± 12	26 ± 7	408 ± 124	43 ± 13.5	26.8 ± 8.9	5 ± 4.2	8 ± 6.5		
Wk 2	1492 ± 150	148 ± 48	293 ± 43	153 ± 22	30 ± 7	467 ± 104	52 ± 14.9	32.7 ± 10.6	79 ± 54	240 ± 88		
Wk 4	1635 ± 419	115 ± 33	347 ± 55	148 ± 11	24 ± 6	645 ± 218	64 ± 20.4	24.5 ± 10				
Controls	1043 ± 82	149 ± 40	199 ± 36	101 ± 12	19 ± 6	54 ± 36 ^c	41 ± 18.3	388 ± 117.6 ^d				

^a Expressed as mg/100 ml (mean ± S.E.).

^b Expressed as 10⁻¹ × the last positive serum dilution (mean ± S.E.).

^c Patients different from controls (*p* < 0.01), but no difference between patient values.

^d Patients different from controls (*p* < 0.05), but no difference between patient values.

Table 2

Effects of bleomycin on cell-mediated immunity

The mean score for 6 patients who were treated with bleomycin and tested for immunocompetence both prior to therapy and after 2 and 4 weeks of bleomycin treatment is compared to normal control scores.

	Immunological test			
	Recall delayed hypersensitivity reaction ^a	Primary delayed sensitivity reaction; KLH ^a	Sheep cell rosettes ^b	Lymphocyte blastogenesis; PHA ^c
Prestudy	6.1 ± 2.0		24 ± 8.5	99 ± 51
Wk 2	5.1 ± 2.3	28.8 ± 17	19 ± 3.0	19 ± 6
Wk 4	8.1 ± 3.0	15.4 ± 5	33 ± 9.9	31 ± 13
Controls	27.7 ± 2.0 ^d	55 ± 19	48 ± 2.6 ^e	112 ± 24

^a Mean diameter of all skin tests with any measurable induration expressed in mm (± S.E.).

^b Mean percentage of lymphocytes forming rosettes (± S.E.).

^c Mean stimulation index calculated by ratio of cpm in stimulated to that in unstimulated controls (± S.E.).

^d Patients different from controls (*p* < 0.001), but no difference between patient values.

^e Patients different from controls (*p* < 0.01), but no difference between patient values.

Table 3

In vitro effects of bleomycin on PHA-induced thymidine incorporation

	In vitro bleomycin concentration			
	No bleomycin	0.0015 mg/ml	0.015 mg/ml	0.15 mg/ml
Unwashed ^a	144 ± 42 ^b	129 ± 25	30 ± 9 ^c	6 ± 2.5 ^d
Washed ^e	234 ± 100	382 ± 105	264 ± 95	131 ± 47

^a Lymphocytes were cultured in the presence of bleomycin for the entire 72-hr culture period.

^b Mean stimulation index calculated as the ratio of cpm in stimulated cultures to cpm in unstimulated controls (± S.E.).

^c Significantly reduced (*p* < 0.05).

^d Significantly reduced (*p* < 0.001).

^e Lymphocytes were cultured with bleomycin for 1 hr and then were washed to remove bleomycin for the remaining culture period.

has been reported by several investigators (1, 5, 16, 21) not to be immunosuppressing in animal studies.

Bleomycin was administered at a high dose by a continuous schedule in this study, which should have produced a maximum potential immunosuppressive effect. As seen in Table 1, there was no immunosuppression of humoral immunity. Previous studies have shown that, following primary vaccination with KLH, patients who are immunosuppressed have significantly lower antibody titers and a prolonged IgM response without IgG antibody production (9, 12). Bleomycin neither depressed antibody titers nor inhibited the conversion to IgG antibody production.

Cellular immunity was also not suppressed by bleomycin treatment. Both recall and primary delayed hypersensitivity reactions were unchanged during the treatment period. Lymphocyte stimulation with PHA was somewhat reduced in treated patients.

Mathé has referred to unpublished studies demonstrating no immunosuppressing effect from bleomycin in man, but

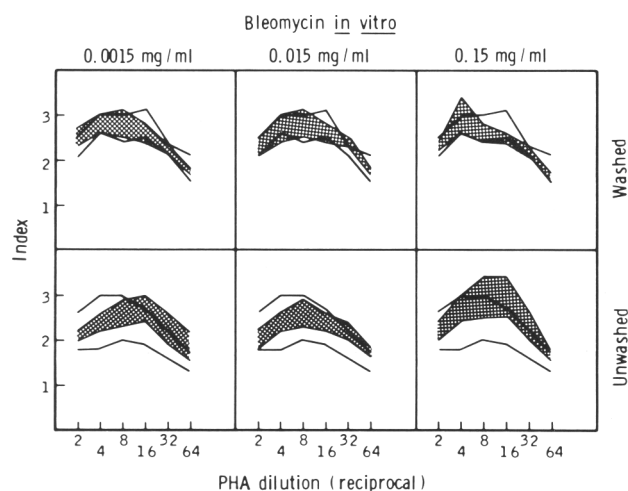


Chart 1. Normal human lymphocytes were stimulated with serial dilutions of PHA. "Unwashed" refers to cultures that were exposed to 3 concentrations of bleomycin for the entire 20 hr of incubation, while "washed" refers to cultures that were incubated in the presence of bleomycin for 1 hr, following which bleomycin was removed. *Solid lines*, mean stimulation index \pm 1 S.E. for each dilution of PHA in control cultures; *shaded area*, mean stimulation index \pm 1 S.E. for bleomycin-treated lymphocytes.

other investigators have shown that bleomycin inhibits lymphocyte stimulation with PHA *in vitro*, by means of DNA synthesis assays to measure lymphocyte reactivity (14, 16, 17).

In the present studies, exposure of PHA-stimulated lymphocytes to bleomycin for 72 hr at a concentration comparable to that achieved in human plasma (0.0015 mg/ml) did not inhibit thymidine incorporation, but a concentration 100-fold higher was inhibitory. The high concentration is comparable to that found in sensitive tumors. However, if bleomycin was removed from the culture medium at 1 hr, simulating plasma clearance of the drug, there was no inhibition of thymidine incorporation even with the highest concentration.

Levy and Kaplan (13) found that [^3H]leucine incorporation by PHA-stimulated lymphocytes was more sensitive than thymidine incorporation for detecting small differences in lymphocyte function, and use of this method enabled them to discriminate immunologically between patients with early and advanced Hodgkin's disease. As shown in Chart 1, there was no inhibition of leucine incorporation in PHA-stimulated lymphocytes, even at the highest concentration of bleomycin. Consequently, we do not interpret the observed inhibition of thymidine incorporation by PHA-stimulated lymphocytes following prolonged exposure to a high concentration of the drug as inhibition of lymphocyte blastogenesis by bleomycin. We interpret the observed decrease in thymidine incorporation by PHA-stimulated lymphocytes following prolonged exposure to high concentrations of bleomycin as a specious indication of inhibited lymphocyte blastogenesis. Bleomycin binds to DNA and causes strand splitting, as well as reduced thymidine incorporation and the leaching of thymidine from DNA (15). *In vitro* studies have demonstrated that bleomycin inhibits thymidine but not amino acid incorporation by *Escherichia*

coli and HeLa cell cultures (18). The inhibition of thymidine incorporation by PHA-stimulated lymphocytes probably reflects biochemical actions of the drug on DNA metabolism, rather than impaired blastogenesis. It occurs only after prolonged incubation of lymphocytes with high concentrations of the drug. Consequently, the failure of high doses of the drug to reduce leucine incorporation with PHA-stimulated lymphocytes indicates that blastogenesis is not inhibited.

We have concluded that bleomycin administered by a high dose continuous schedule for 4 weeks does not suppress either humoral or cellular immunity in man. It is particularly interesting that normal lymphocyte function as measured by immunocompetence studies is not affected by bleomycin, while malignant lymphoma is responsive to bleomycin. Umezawa *et al.* have isolated a deaminating enzyme that inactivates bleomycin, and they have found this enzyme in low concentrations in cells that respond to bleomycin, suggesting one mechanism by which selective toxicity may be achieved (10, 19). Studies to determine inactivating enzyme levels in normal and malignant lymphocytes are under way in an attempt to understand the effects of bleomycin in humans.

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REFERENCES

1. Andrews, E. J. An *in Vivo* Evaluation of the Immunosuppressive Action of Bleomycin. *Cancer Res.*, 32: 1993-1994, 1972.
2. Blum, R. H., Carter, S. K., and Agre, K. A Clinical Review of Bleomycin. A New Antineoplastic Agent. *Cancer*, 31: 903-913, 1973.
3. Bruckner, H. W., Mokyr, M. B., and Mitchell, M. S. Effect of Imidazole-4-carboxamide, 5-(3,3-Dimethyl-1-triazeno) on Immunity in Patients with Malignant Melanoma. *Cancer Res.*, 34: 181-183, 1974.
4. Cheema, A. R., and Hersh, E. M. Patient Survival after Chemotherapy and Its Relationship to *in Vitro* Lymphocyte Blastogenesis. *Cancer*, 28: 851-855, 1971.
5. Dlugi, A. M., Robie, K. M., and Mitchell, M. S. Failure of Bleomycin to Affect Humoral or Cell-mediated Immunity in the Mouse. *Cancer Res.*, 34: 2504-2507, 1974.
6. Fujita, H. Comparative Studies on the Blood Level, Tissue Distribution, Excretion and Inactivation of Anticancer Drugs. *Japan. J. Clin. Oncol.*, 1: 151-162, 1971.
7. Halterman, R. H., and Leventhal, B. G. Enhanced Immune Response to Leukemia. *Lancet*, 2: 704-705, 1971.
8. Hersh, E. M., and Freireich, E. S. Host Defense Mechanisms and Their Modifications by Cancer Chemotherapy. *In: H. Busch (ed.), Methods in Cancer Research*, Vol. 4 pp. 355-451. New York: Academic Press, Inc., 1968.
9. Hersh, E. M., Whitecar, J. P., McCredie, K. B., Bodey, G. P., and Freireich, E. S. Chemotherapy, Immunocompetence, Immunosuppression and Prognosis in Acute Leukemia. *New Engl. J. Med.*, 285: 1211-1216, 1971.
10. Hopsu, V. K., Makinen, K. K., and Glenner, G. G. Purification of a Mammalian Peptidase Selective for N-Terminal Arginine and Lysine Residues: Aminopeptidase B. *Arch. Biochem. Biophys.*, 114: 557-566, 1966.

11. Lehane, D. E., and Lane, M. Immunocompetence in Advanced Cancer Patients Prior to Chemotherapy. *Oncology*, 30: 458-466, 1975.
12. Lehane, D. E., Lane, M., and Wardle, M. Immunocompetence of Head and Neck Cancer Patients Receiving Methotrexate (MTX) by Two Different Schedules. *Proc. Am. Soc. Clin. Oncol.*, 15: 190, 1974.
13. Levy, R., and Kaplan, H. S. Impaired Lymphocyte Function in Untreated Hodgkin's Disease. *New Engl. J. Med.*, 290: 181-186, 1974.
14. Mathé, G., and Hayat, M. Study of the Clinical Efficiency of Bleomycin in Human Cancer. *Brit. Med. J.*, 2: 643-645, 1970.
15. Muller, W. E. G., Yamazaki, Z., Bretter, H. J., and Zahn, R. K. Action of Bleomycin on DNA and RNA. *European J. Biochem.*, 31: 518-525, 1972.
16. Ohno, R., Nishwaki, H., Kawashima, K., Tadaaki, U., Hirano, M., Miura, M., and Yamada, K. Lack of Immunosuppressive Effect of Bleomycin on the Primary Response of Mice to Sheep Red Blood Cells. *Gann*, 62: 267-274, 1971.
17. Tisman, G., Herbert, V., Go, L. T., and Brenner, L. Marked Immunosuppression with Minimal Myelosuppression by Bleomycin *In Vitro*. *Blood*, 41: 721-726, 1973.
18. Umezawa, H., Ishizuka, M., Maeda, K., and Takeuchi, T. Studies of Bleomycin. *Cancer*, 20: 891-895, 1967.
19. Umezawa, H., Takeuchi, T., Hori, S., Sawa, T., and Ishizuka, M. Studies on the Mechanism of Antitumor Effect of Bleomycin on Squamous Cell Carcinoma. *J. Antibiot.*, 25: 409-420, 1972.
20. Wybran, J., and Fundenberg, H. Thymus-Derived Rosette-Forming Cells in Various Human Disease States: Cancer, Lymphoma, Bacterial and Viral Infections, and Other Diseases. *J. Clin. Invest.*, 52: 1026-1032, 1973.
21. Yamaki, H., Tanaka, N., and Umezawa, H. Effects of Several Tumor-Inhibitory Antibiotics on Immunological Responses. *J. Antibiot.*, 22: 315-321, 1969.