ENHANCEMENT OF ANTIBODY SYNTHESIS BY
6-MERCAPTOPURINE*

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PLATE 31

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The antimetabolite six-mercaptopurine (6-MP) is a powerful immunosuppressive drug. In experimental animals it depresses the synthesis of circulating antibodies (1), inhibits delayed hypersensitivity (2), and retards the rejection of allografts (3). On the basis of these findings, the treatment of a variety of immunological diseases and attempts to delay the rejection of human allografts have been undertaken with 6-MP or its derivates, thioguanine and azathioprine (4). Increasing clinical experience with these agents indicates, however, that an incomplete understanding of how they act hampers their most effective use. For example, though it is often assumed that the clinical results obtained with 6-MP are due to suppression of immunity, other effects of this antimetabolite, such as inhibition of inflammation (5, 6), cannot be discounted. The purpose of this report is to describe an additional and paradoxical action of 6-MP, the enhancement of antibody synthesis.

These experiments were prompted by the observation that 6-MP treatment can cause hypertrophy of lymphoid tissue (7). In rabbits, hyperplasia of the spleen and lymph nodes appeared within 2 days after the last dose of a 1 wk course of 6-MP (10 mg/kg/day) and gradually diminished during the succeeding 3 wk. Five days after such a course of 6-MP the lymphoid hyperplasia was at its peak. The spleen and lymph nodes were grossly enlarged and on microscopic examination they were seen to contain many follicles with greatly enlarged germinal centers. Numerous primitive cells resembling hemocytoblasts were present. There were, however, no morphologic signs of antibody formation: neither tingible bodies nor mature plasma cells were found (Fig. 1).

In the following experiments, the immune responses of rabbits recovering from a 7 day course of 6-MP were tested and compared with antibody produc-

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tion in animals given 6-MP according to several other schedules. A wide range of effects on antibody synthesis, from marked enhancement to profound suppression, was found.

**Materials and Methods**

**Animals.**—Adult New Zealand white rabbits, obtained from a local supplier, housed under standard conditions, and fed Purina Rabbit Chow and water ad lib. were used.

**Antigen.**—Bovine gamma globulin (BGG, Pentex) was dissolved in 0.15 M NaCl to the desired concentration. I^{131}-labeled BGG was prepared by the Helmkamp procedure (8). Aggregated BGG was prepared by heating a solution of BGG in a water bath at 63°C for 30 min.

**Antibody Titration.**—Bleedings were obtained at frequent intervals, particularly during the early phases of the response, and antibody titrations were done by means of the tanned red blood cell technique (9). The results, which were reproducible within ±1 serial dilution, were converted to log₂ units for purposes of graphic presentation. IgM (19S) and IgG (7S) antibodies were discriminated on the basis of sensitivity to 2-mercaptoethanol (2-ME) (10). We (11) previously showed that anti-BGG antibodies obtained from both normal and 6-MP-treated rabbits which were inhibited by 0.1 M 2-ME were found in the bottom layers of an ultracentrifuged sucrose gradient and in the first protein peak after chromatography of whole serum on Sephadex G-200. In contrast, antibodies insensitive to the action of 0.1 M 2-ME were found in the upper layers of the gradient and in the second protein peak of chromatographed serum. In agreement with others (12, 13), the larger, heavier 2-ME-sensitive antibodies were classified as IgM and the smaller, lighter 2-ME-resistant antibodies were termed IgG.

The clearance from the circulating blood of I^{131}-tagged BGG was determined by measuring radioactivity of serum samples in a well-type scintillation counter according to methods previously described (1).

**6-Mercaptopurine.**—6-MP was freshly prepared just before use by dissolving an appropriate amount in 1.0 M NaOH, then diluting with 0.15 M saline to the desired concentration (10 mg/ml). Each animal received 1 ml of the dissolved drug per kg subcutaneously. Regardless of timing schedules (with reference to the day of antigen administration), all test rabbits received 10 mg/kg of 6-MP daily for 1 wk. This dosage of the drug was not associated with overt signs of toxicity such as diarrhea and weight loss.

**RESULTS**

Three different schedules of 6-MP administration and three different levels of antigen dosage were studied systematically (Text-figs. 1, 2, and 3). Each test animal was given 6 MP, 10 mg/kg/day for 7 days. Two, 5, or 20 days after the last injection of the drug they received a single intravenous injection of either 0.02, 2.0, or 200 mg of BGG. Control rabbits were given only the antigen. There were thus nine groups of test animals and three groups of control rabbits. Each group comprised 4 to 8 animals.

Two days after the last dose of a 1 wk course of 6-MP, enhancement of antibody synthesis was found in rabbits given 0.02 mg and 2.0 mg of BGG, but not in the group which had received 200 mg of BGG (Text-fig. 1). A dose of 0.02 mg of BGG failed to evoke a primary immune response in most of the control rabbits. In contrast, a brisk antibody response was elicited in all the test animals given this dose of antigen. Most of the hemagglutinating anti-BGG
TEXT-Fig. 1. Antibody responses when BGG was given 2 days after the last dose of a 1 wk course of 6-MP. In this and succeeding charts, titers of control animals are shown by lines connected by closed circles; titers of 6-MP-treated rabbits are indicated by lines connected by open circles. The dose of antigen is shown above each vertical panel. In all instances, antigen was given intravenously on day 0.
Text-Fig. 2. Antibody responses when BGG was given 5 days after the last dose of a 1 wk course of 6-MP. See legend to Text-fig. 1.
Text-Fig. 3. Antibody responses when BGG was given 20 days after the last dose of a 1 wk course of 6-MP. See legend to Text-fig. 1.
antibodies produced by these rabbits was IgM. Rabbits given 2.0 mg of BGG 2 days after the last dose of 6-MP also showed exaggerated antibody responses. In contrast to animals receiving the lower dosage of antigen, both the IgM and IgG responses were enhanced in this group of rabbits.

Five days after the last dose of 6-MP, when lymphoid hypertrophy has been found to be at its peak (7), all groups of test animals showed enhancement of the immune response (Text-fig. 2). This was particularly evident in rabbits given 0.02 and 2.0 mg of antigen. Enhancement of both IgM and IgG responses occurred in all groups. In animals receiving 0.02 and 2.0 mg of BGG there was a distinct shortening of the induction time (the period between antigen administration and the first appearance of serum antibody), and in some rabbits circulating antibodies could be detected within 12 hours of antigen administration.

When antigen was administered 20 days after the last dose of 6-MP, unequivocal enhancement of total antibody synthesis occurred only when 0.02 mg of BGG was given (Text-fig. 3). Average peak titers of total antibody in the group given 2.0 mg of BGG were similar in both test and control groups (8.0 and 6.5 respectively), but hemagglutinating IgG antibodies appeared earlier in the test group (average, 5.0 days) than in the control group (average, 9.3 days). The average peak titer of IgM antibody was higher in the test group given 200 mg of BGG (7.0) than in the corresponding control group (4.4).

The following experiments were carried out in order to determine whether or not the processes of antigen capture and priming, important elements of the primary immune response, are involved in the enhancement of antibody production by 6-MP. The delineation of antigen capture was attempted in two ways: measurement of the rate of clearance of $^{125}$I-tagged BGG from the circulating blood, and of antibody production in response to a particulate antigen. Effects on the priming mechanism were examined by studies of the secondary response.

The rates of clearance of $^{125}$I-tagged BGG (total concentration of radioactive BGG plus carrier antigen, 2.0 mg/ml; each rabbit received an intravenous dose of 1.0 ml) were estimated in 4 normal rabbits and in 4 animals tested 5 days after the last dose of a 1 wk course of 6-MP, 10 mg/kg/day. The results were similar in the two cases: the average time of appearance of the immune clearance phase was 7.5 days in control rabbits; in test animals it was 7.2 days.

In the next experiment, heat-aggregated BGG was employed as the antigenic stimulus on the supposition that if the 6-MP treatment led to activation of the macrophage system, administration of this particulate form of antigen should lead to a highly exaggerated immune response. Two groups of rabbits were given 6-MP, 10 mg/kg/day for 7 days; 5 days after the last dose of 6-MP, one group received 1.0 ml of a heat-aggregated suspension of BGG, calculated to contain 2.0 mg/ml. The other group was given 2 mg of BGG dissolved in saline.
The results are shown in Text-fig. 4. No additional enhancement of antibody synthesis was found in the rabbits given the particulate form of BGG. It may be noteworthy that the average induction time in the group given BGG in solution was 2.0 days and that in the rabbits receiving aggregated BGG was 4.4 days.

Possible effects of 6-MP on the priming mechanism were studied by examining the secondary immune responses of normal rabbits and of rabbits whose primary immune response had been exaggerated by pretreatment with 6-MP.

Text-fig. 4. Antibody responses when BGG was given either in solution or as a particulate suspension (heat-aggregated), 5 days after the last dose of a 1 wk course of 6-MP.

Two control groups, one given two doses of 2.0 mg of BGG 6 weeks apart, the other challenged in the same way with two doses of 0.02 mg of BGG, were compared with two corresponding test groups which had received their first antigenic challenge 5 days after the last dose of a 1 wk course of 6-MP. 6-MP was not given at any other time. The results are shown in Text-fig. 5. The secondary responses of test and control rabbits given two injections of 2.0 mg of BGG were virtually identical. Antibody titers in test rabbits rechallenged with 0.02 mg of BGG were higher than those in the corresponding controls. However, the drug-treated rabbits had an average titer of approximately 4 log₅ units of antibody at the time of the second stimulus, whereas the control rabbits had no detectable antibody. Since this value of 4 log₅ units probably represented a steady state of antibody synthesis induced by the first injection of
antigen, it was subtracted from each of the values obtained in the drug-treated animals during the secondary response. This resulted in no difference between control and test groups.

In the following experiments, the timing schedules of 6-MP which produced enhancement of antibody synthesis were compared with four other schedules of 6-MP administration. The only variable was the timing of drug administration with respect to the day of antigenic challenge. The first dose of the 7 day course of 6-MP was moved forward progressively from 12 days before the antigenic challenge to 6 days after administration of BGG. Text-fig. 6 shows the results when 2.0 mg of BGG were used; a similar series of experiments was carried out in rabbits challenged with 0.02 mg of BGG and is shown in Text-fig. 7. With either dose of antigen, maximum enhancement of antibody synthesis was found when 6-MP was given on days -12 to -15 (the day of antigen administration counting as 0); maximum suppression of the immune response occurred when the first dose of 6-MP coincided with the day of antigen adminis-
Text-Fig. 6. Antibody responses to 2.0 mg BGG as influenced by a standard course of 6-MP (10 mg/kg daily for 1 wk), given at various times. The hatched bars indicate the timing of 6-MP administration. The vertical arrows show the day of antigen injection. A and B show enhancement of antibody synthesis. In C, the induction period was prolonged, but average peak antibody titers were eventually equal to those of control animals. D and E show profound suppression of antibody synthesis. In F, antibody production was only partially inhibited.
Intermediate degrees of enhancement or suppression were seen with the other schedules.

**DISCUSSION**

The results of these experiments demonstrate that a wide spectrum of effects, ranging from increased antibody formation to complete inhibition of the immune response, can be elicited by treatment with 6-MP. Suppression of the
primary immune response, the first of these effects to be recognized, hinges on a series of important early events which are activated by the contact of lymphoid tissue with antigen (4). In contrast, the enhancement of antibody formation by 6-MP depends upon effects initiated by the drug before the encounter between lymphoid tissue and antigen.

Any explanation of the adjuvant effect of 6-MP must take into consideration two apparently divergent observations: (a) the main effect of the drug is cytotoxicity, and (b) pronounced lymphoid hyperplasia can occur after treatment with this antimetabolite. We propose that nucleic acids released from cells injured by 6-MP could account for both the hyperplastic lymphoid tissue and the enhanced production of antibodies. In the ensuing paragraphs we shall analyze the following interpretation of the adjuvant effect of 6-MP: (a) The 1 wk course of drug treatment led to the death of various cells. (b) On discontinuation of the suppressive drug, nucleic acids derived from injured cells initiated stimulation of lymphoid tissue. The result was hyperplasia of lymph nodes and spleen, which reached a peak 5 days after the last dose of 6-MP. (c) Introduction of antigen at that time was associated with a rapid and maximal response by the expanded and activated lymphoid tissue.

Six-MP affects cells by blocking the conversion of inosinic acid to adenylic acid (14). Since adenylic acid is essential in the biosynthesis of nucleic acids, proteins, carbohydrates, and lipids, the specific biochemical lesion induced by 6-MP leads to widespread and ultimately fatal disorders in cellular metabolism. It is reasonable to assume that, following injury by this antimetabolite, the contents of dying and dead cells, including nucleic acids, are released into the local environment. The importance of nucleic acids is stressed because these cell constituents can enhance the production of antibodies (15, 16). To the best of our knowledge, no other cell breakdown product has this property. Since enzymatically digested DNA and RNA also enhance antibody synthesis (15–18), hydrolysis by local nucleases would not necessarily abolish any adjuvant effects of nucleic acids deposited in situ.

Following their release from 6-MP-injured tissue, nucleic acids or their degradation products could be taken up by living cells. It is known that the DNA of lymphocytes is reutilized by cells of intestinal crypts and regenerating liver (19) and that polynucleotides can be incorporated directly into lymphocytes (20) and strain L cells (21). Even entire chromosomes can be taken up by mammalian cells (22). Nucleic acids or nucleic acid digests could, after their incorporation into cells, have profound effects, since they are capable of stimulating cell division (23), protein synthesis (24), and enhanced growth of cells (25, 26).

An increase only in the total mass of lymphoid tissue in 6-MP-treated rabbits should have resulted in enhancement of antibody synthesis at each dosage of antigen used. This was not the case; in the drug-treated rabbits large doses of
antigen provoked only slightly more antibody synthesis than small doses. Text-fig. 8 shows an experiment in which antigen doses differing by 5 orders of magnitude evoked similar peak titers of antibody in 6-MP–treated rabbits. Thus, in addition to hyperplasia, a qualitative change involving a specialized lymphoid cell seems likely. Several findings might indicate the nature of this cell: (a) There was no evidence of accelerated clearance of antigen from the circulating blood; (b) challenge with heat-aggregated (particulate) BGG was not associated with enhancement above that found with BGG in solution; (c) the secondary responses of rabbits whose primary responses were augmented by 6-MP were normal; (d) large numbers of hemocytoblasts were present in the lymphoid tissues at the time of maximum enhancement. The first two observations suggest that systems involved in the capture of antigen (macrophages)
were not influenced by the 6-MP treatment. The third finding means that immunological memory was not affected. These three groups of results suggest the participation of a cell capable of direct stimulation by antigen and lacking a memory mechanism. Such a cell might be the hemocytoblast (27, 28), the type of cell actually observed in large numbers 5 days after the last dose of a 1 wk course of 6-MP.

The experimental results concerned with antigen dosage seem consistent with a clonal selection theory of antibody formation. It is conceivable that nucleic acids from dead cells could stimulate a precursor cell (lymphocyte?) to differentiate into a hemocytoblast in a manner analogous to the action of phytohemagglutinin (29). Assuming that a nearly synchronous transformation of precursor lymphocytes to antigen-sensitive hemocytoblasts occurred, an intravenous dose of 0.02 mg of BGG would define the maximum responsiveness of the "anti-BGG clone." Higher doses of antigen did not result in substantially more antibody synthesis because of the physical limits of that clone.

In addition to 6-MP, many other cytotoxic agents are capable of enhancing antibody synthesis, including X-irradiation (30, 31), 5-fluoro-2-deoxyuridine (32), uracil mustard (33), cyclophosphamide (33), cortisone (34), penicillamine (35), colchicine (36), and endotoxin (37). In mice, whole body X-irradiation, prednisone, methotrexate, and 6-MP markedly aggravated allogenic disease (38), an immunologic disorder caused by the graft versus host reaction. It has been previously proposed that the adjuvant effects of some of these agents are mediated by nucleic acids released from injured cells (32, 39, 40). The observations that nucleic acids and nucleic acid digests can restore antibody formation in X-irradiated animals (15, 16, 41) and in 6-MP-treated rabbits (42), that the nuclear contents of spleen cells accelerate allogenic disease (43), and that oligonucleotides enhance antibody formation in mice (17, 18) are all consistent with this interpretation of the adjuvant effects of cytotoxic agents.

The generality of the phenomenon of increased antibody synthesis resulting from treatment with agents also possessing immunosuppressive and cytotoxic effects has obvious clinical significance. The "triggering" of autoimmune hemolytic anemia in patients with lymphosarcoma or chronic lymphocytic leukemia by X-rays and alkylating agents has already been described (44). Whether or not the elucidation of this bimodal effect in experimental animals will affect current trends in immunosuppressive therapy will naturally depend on the results of clinical investigations.

SUMMARY

The administration of 6-MP to rabbits led to either suppression or enhancement of antibody production, depending on when the drug was given in relation to the antigenic challenge. Maximum enhancement of antibody synthesis was found when a small dose of BGG was administered 5 days after the last dose of a
1 wk course of 6-MP. There were no indications that the macrophage system or immunological memory was affected in animals with augmented antibody synthesis. It was proposed that enhancement of antibody production by 6-MP was due to nucleic acids released from cells killed or injured by the drug. It was suggested that lymphocytes incorporating these nucleic acids were transformed into specialized cells capable of direct and immediate stimulation by antigen and lacking immunological memory (hemocytoblasts). A relatively small dose of antigen was apparently capable of stimulating all the hemocytoblasts representing a given clone, with the result that large amounts of antibody rapidly appeared in the serum.

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deoxyuridine on the primary antibody response of the BALB mouse to a purified protein antigen, *J. Immunol.*, 1963, 91, 266.


EXPLANATION OF PLATE 31

Figs. 1 a to d. Histologic appearances of rabbit lymph nodes 5 days after the last dose of a 1 wk course of 6-MP, 10 mg/kg daily.

Fig. 1 a. Low power view, showing numerous follicles with enlarged germinal centers. Hematoxylin and eosin stain. ×45.

Fig. 1 b. Numerous blast cells in the medulla, characteristically grouped around and sometimes filling lymphatic channels (arrows). Giemsa stain. × 450.

Figs. 1 c and d. Additional examples of blast cells (classified as hemocytoblasts). × 450. c, Giemsa stain; d, hematoxylin and eosin stains.