THE RELEASE OF SPECIFIC BACTERICIDAL ANTIBODIES BY ENDOTOXIN*

BY J. GABRIEL MICHAEL,‡ PH.D.

(*From the Rheumatic Fever Research Unit, House of the Good Samaritan, Children's Hospital Medical Center, and the Department of Pediatrics, Harvard Medical School, Boston*)

Administration of endotoxin to mice is followed by a sudden increase in the bactericidal activity of the animal's serum. The increase in serum activity is chiefly due to a rise in titer of specific antibodies in the circulation (1). In experiments reported here we attempted to determine whether this increase in bactericidal antibodies is the result of a release of preformed globulins from antibody-forming cells or whether endotoxin stimulates antigen-sensitive cells to rapid production of antibodies.

In our studies we utilized Jerne's agar plaque technique (2). This technique permits recognition of antibody-forming cells which release the antibodies into their surroundings. Although this technique was developed only recently, it has been successfully employed by many investigators. With this method it is possible to identify hemolysin and bactericidin-forming cells (3–5).

Materials and Methods

Agar Plaque Technique.—Mouse spleen cell suspensions, agar plates, and liquid media were prepared as described by Jerne (2). In brief, mouse spleen cells were suspended in medium 199 in concentration of about 1×10^8 cells per ml. One-tenth ml of the cell suspension was placed in 2.0 ml of 0.7% Noble agar prepared in medium 199. An inoculum of 1×10^8 bacteria of a serum-sensitive strain of *Escherichia coli* 0127:B8 in 0.1 ml was added to each agar tube. The tubes were maintained in a 45°C water bath. After thorough mixing, the contents of the tubes were poured into Petri dishes and incubated for 1 hr at 37°C. Following incubation, 1.5 ml of a 1:5 dilution of fresh human cord serum (complement source) was poured over the surface, and the plates were reincubated for 6 hr at 37°C. During this time the bacteria multiplied sufficiently so that clear plaques in the agar were recognized around bactericidin-forming cells.

Bactericidal Antibodies.—Titers of bactericidal antibodies in the mouse sera were measured by a method described in several publications (6, 7). The titers represented the highest dilutions of mouse serum which, in conjunction with complement (fresh human cord serum), kills 75% of an inoculum of 10,000 bacteria within 1 hr.

The bactericidal index showed the ratio between antibody titers of treated and untreated mice.

Animals.—6-wk-old female white Swiss mice, weighing about 30 g each, were supplied by the Charles River Farms Breeding Laboratories, Brookline, Massachusetts.

Endotoxins.—The lipopolysaccharidic antigens (endotoxins), utilized in these experiments, were prepared in this laboratory by water-phenol extraction of bacteria. These substances were prepared from four bacterial species; *Escherichia coli 0127:B8, Pseudomonas aeruginosa, Salmonella typhi,* and *Shigella flexneri.* The results obtained with the last three preparations were essentially the same and therefore only experiments in which *E. coli* and *Pseudomonas* antigens were used are described in this report.

Preparation of 6-Mercaptopurine (6-MP) Solutions.—6-mercaptopurine ("Purinethol" brand) was generously donated by Burroughs, Wellcome and Company, Tuckahoe, New York and was prepared daily in 8 N NaOH. It was diluted to the desired concentration with sterile saline and adjusted to pH by titration with diluted HCl.

TABLE I

Effect of Endotoxin on Bactericidin-Producing Spleen Cells and Titers of Bactericidal Antibodies in Mice

Time After i.v. injection of 50 μg *Pseudomonas* endotoxin	No. of plaques per spleen	No. of plaques per million spleen cells	Relative serum antibody levels for *E. coli* 0127:B8
0 hr	660	6.7	1.0
3 hr	80	0.5	4.2
6 hr	210	1.5	5.0
12 hr	425	3.4	3.7
18 hr	707	7.4	3.9
48 hr	2100	15.0	2.6
72 hr	5620	37.7	1.0
96 hr	970	10.0	1.0
7 days	714	8.1	1.0

Average total number of cells per spleen 1–3 $\times$ 10^8 (3 animals per group).

RESULTS

Effect of Endotoxin on Count of Plaque-forming Cells.—We have shown that normal mice have high titers of bactericidal antibodies against *E. coli* 0127:B8 and, therefore, we anticipated that the spleens of these animals would contain a reasonable number of bactericidin-producing cells. This expectation was fulfilled as shown in Table I. The number of plaque-forming cells per spleen was about 700, and there was only slight variation in this count among the animals tested.

The effect of endotoxin on the plaque-forming cells was tested as follows: mice were injected intravenously with 50 μg of *Pseudomonas* endotoxin per animal and, after varied intervals, groups of 3 mice were bled and their spleens removed. 3 hr after the injection of endotoxin the mice developed increased levels of specific bactericidal antibodies (Table I). This increase in antibodies was noticeable for 48 hr, after which the titer dropped to the level present at the time of injection of endotoxin. In contrast, a few hours after the administration

of endotoxin, the number of plaque-forming cells sharply declined. The plaque count remained low for only 12 hr after which it returned to the level found in untreated animals. The number of the cells then continued to rise reaching the maximum count at 72 hr. We will deal with this increase in the later part of the results. The apparent decrease in the number of plaque-forming cells following the injection of endotoxin might either be the result of the destruction of cells or caused by the inability of the cells to produce or release antibodies. To examine this question, we used 6-mercaptopurine to inhibit cellular division. We reasoned that, if endotoxin would cause the destruction of antibody-forming
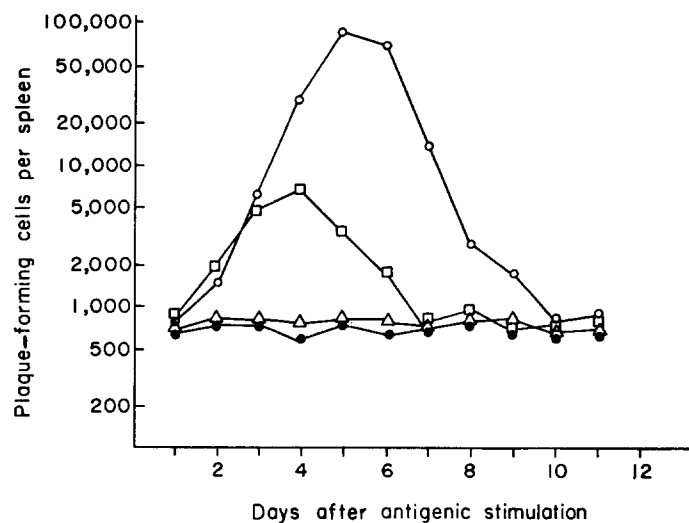

FIG. 1. Counts of plaque-forming cells in mouse spleens after injection of animals with *Escherichia coli* 0127 somatic antigen and 6-mercaptopurine. Each point represents averages from 3 mice. O, antigen only; ●, 6-MP only; △, antigen + 6-MP simultaneously; and □, antigen + 6-MP delayed for 3 days.

cells, in the presence of 6-MP the production of new cells would be prevented and the plaque count would therefore remain low.

Suppression of Immune Response by 6-MP.—To determine whether 6-MP can inhibit the multiplication of bactericidin-forming cells, we tested the effect of this immunosuppressive drug on an immune response. Mice were injected intravenously with 10 μg of *E. coli* 0127:B8 antigen and then they were given a daily intravenous injection of 6-MP in a dose of 150 mg per 1000 g of animal. In one group of mice the administration of 6-MP was started simultaneously with the antigen; in the second group 6-MP treatment was initiated 3 days after injection of the antigen. Of the two control groups of animals, one was injected with antigen alone and the second was injected only with 6-MP.

Fig. 1 graphically summarizes the responses to the modified antigenic stimuli as expressed in counts of plaque-forming cells in the spleens of the mice. Fig. 2 gives the results expressed as antibody titers. When antigen was given alone there was a rapid exponential increase in the number of bactericidin-forming cells and in the titer of bactericidal antibodies. The peak response, averaging 80,000 plaque-forming cells per spleen, was reached on the 5th day and the maximum titer of bactericidins was attained on the 7th day. Mice injected with antigen and 6-MP simultaneously failed to exhibit any antibody response; the number of bactericidin-forming cells in the spleens of these animals remained the same as that in the untreated control group. The most revealing results were obtained
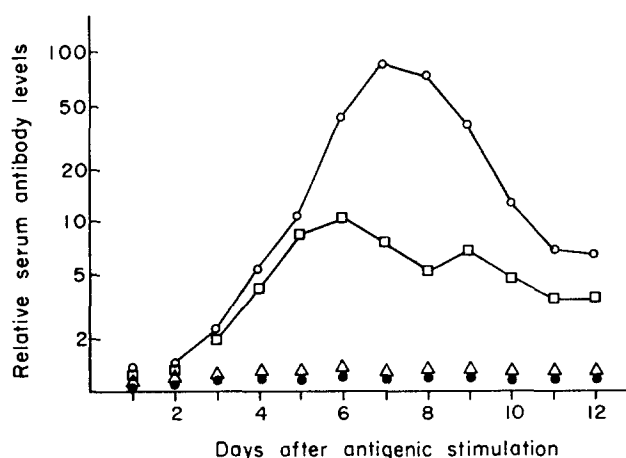

FIG. 2. Relative serum antibody levels in mice after injection of animals with *Escherichia coli* 0127 somatic antigen and 6-mercaptopurine. Each point represents averages from 3 mice. Symbols as in Fig. 1.

with spleens and sera of mice given 6-MP 3 days after the antigen (Figs. 1 and 2). After administration of the antigen there was an increase in plaque-forming cells and in antibody, but following treatment with 6-MP both the cellular and the humoral responses were arrested. These data indicated that 6-MP suppressed response to somatic antigen of Gram-negative bacteria, and that the inhibition of antibody formation was achieved by preventing the proliferation of cells responding to antigenic stimulation.

Effect of 6-MP on Release of Bactericidal Antibodies.—Since 6-MP inhibited the multiplication of bactericidin-forming cells, it was tested to see if it would modify the nonspecific response of these cells to endotoxin. Mice were injected intravenously with *Pseudomonas* endotoxin (50 μg) and with 6-MP (4.5 mg) and groups of animals were then bled and sacrificed to determine their antibody titers and their spleen plaque counts. The tested mice were injected daily with

4.5 mg of 6-MP. As shown in Table II, the initial response of the plaque-forming cells was not affected by 6-MP. The number of the bactericidin-forming cells dropped and rose in the same way as it did in the absence of 6-MP. However, the increase in cell count which was previously observed at 72 hr did not occur at this time. These results led to the conclusion that under our experimental conditions endotoxin did not cause the destruction of antibody-forming cells.

Stimulation of Bactericidin and Hemolysin-Forming Cells.—We observed that for a limited period of time, about 72 hr after the injection of endotoxin, the count of bactericidin-forming cells was increased about tenfold (Table I).

TABLE II

Effect of 6-Mercaptopurine on Endotoxin-Treated Mice as Expressed in Counts of Bactericidin-Producing Spleen Cells and Titers of Bactericidal Antibodies

Time After i.v. injection of 50 μg *Pseudomonas* endotoxin*	No. of plaques per spleen	No. of plaques per million spleen cells	Relative serum antibody levels for *E. coli* 0127:B8
0 hr	760	7.0	1.0
3 hr	47	0.3	3.6
6 hr	109	1.0	4.5
12 hr	217	2.3	4.0
18 hr	641	6.1	3.2
48 hr	711	7.1	2.8
72 hr	660	8.0	1.2
96 hr	820	7.4	1.0
7 days	751	6.9	1.0

Average total number of cells per spleen 1–3 × 10^8 (3 animals per group).

* First dose of 6-MP injected simultaneously with endotoxin.

A similar observation with sheep hemolysin-forming cells was made recently by Heuer and Pernis (8); and we have confirmed their results. Since the same endotoxin had stimulated the proliferation of cells that produced either one of two types of antibodies, it was of interest to determine whether both antibodies were produced by one type of cell. Mice were intravenously injected with 50 μg *Pseudomonas* endotoxin, and 72 hr later their spleens were removed. The spleen cell suspensions were incorporated into soft agar which contained both sheep red cells and *E. coli* 0127:B8 bacteria (for details see Materials and Methods). After 1 hr of incubation at 37°C, the surface of the plates was flooded with 1.5 ml of fresh human cord serum (dilution 1:5) and incubated at 37°C for 30 min. Plaques appearing at this time indicated the location of hemolysin-forming cells, and they were marked on the bottom of the plates. The Petri dishes were returned to the incubator for an additional 6 hr at which time numerous new plaques became visible showing the location of bactericidin-forming cells. We found that the plaques representing hemolysin-forming cells

appeared at sites completely different from those of bactericidin-producing cells. Thus, we demonstrated that endotoxin stimulated proliferation of two types of antibody-forming cells.

DISCUSSION

It has been reported that endotoxin can be toxic for cells of the reticulo-endothelial system (9). Other investigations showed endotoxin to be an adjuvant capable of increasing antibody response to protein antigens (10). When endotoxin and a protein antigen were given together, a marked proliferation of cells in the spleen folicles occurred. In this study, we investigated whether endotoxin would exert a cytotoxic or stimulatory effect on bactericidin-forming cells.

We have recently demonstrated that the titers of bactericidal antibodies to Gram-negative bacteria are markedly increased a few hours after the administration of endotoxin. Because of the presence of antigenic stimuli, these types of antibodies were produced by the animals prior to the injection of endotoxin. Circulating bactericidal antibodies are being manufactured by cells of lymphatic tissues in different organs of the body, but we tested only the spleen because it is an abundant and convenient source of these cells.

6-mercaptopurine is widely used as an immunosuppressive drug, but its mode of action has not been clearly established (11). We believe that the data presented in this study provide new, useful information in this direction. When 6-MP was administered simultaneously with the antigen neither proliferation of plaque-forming cells nor an increase in titer of bactericidal antibodies was evident. Upon delaying the administration of 6-MP for 3 days after the injection of antigen, there was an initial proliferation of the antibody-forming cells halted by the immunosuppressive drug. These data support the suggestion that 6-MP inhibits the immune response by suppressing the multiplication of the responding cells.

6-MP did not modify the counts of plaque-forming cells following the injection of endotoxin and, therefore, it seems apparent that under our experimental conditions these cells were not destroyed. The fact that the count of bactericidin-forming cells remained low only for a few hours also argues against the possibility that endotoxin would cause destruction and subsequent recovery of these cells. It is hardly conceivable that new generations of antibody-forming cells would be produced in such a short time. We assume, therefore, that the administration of endotoxin precipitates the release of preformed antibodies without apparent injury to antibody-forming cells. The temporary reduction in the number of plaque-forming cells probably reflects their inability to release more bactericidal antibodies for a short time. It remains to be determined whether endotoxin can enhance the release of antibodies from the cells of highly

immunized animals and also which type of antibodies are susceptible to this release mechanism.

Finally, it is worthwhile to consider the possible analogy between the effect of endotoxin on antibody-forming cells and the influence of endotoxin on lysosomes. It has been shown that endotoxin renders lysosomes more permeable to the enzymes which they contain and makes them more susceptible to lysis (12). It is therefore quite plausible that endotoxin may affect the permeability of antibody-storing cells and trigger the release of antibodies.

SUMMARY

Studies presented in this report are concerned with the effect of endotoxin upon the release of bactericidal antibodies as determined by the agar plaque technique. We found that shortly after the injection of mice with small amounts of endotoxin the number of plaque-forming cells in the spleen was substantially reduced and remained low for about 12 hr. At the same time the titer of specific bactericidal antibodies in the circulation was increased. 6-mercaptopurine was shown to be an effective suppressor of the immune response by inhibiting the multiplication of bactericidin-forming cells. However, administration of this drug did not modify the initial responses of antibodies and plaque-forming cells to endotoxin. Thus, we postulate that endotoxin does not induce destruction and subsequent reconstitution of bactericidin-forming cells, but that the observed changes represent a release of preformed antibodies from competent cells. For a limited time, thereafter, these cells become inactive, but soon resume their normal function of releasing bactericidal antibodies.

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