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Effect of 6-Mercaptopurine on Endotoxin Tolerance *

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Hyperfunction of the reticuloendothelial system (RES) has been considered to be the major mechanism for the development of endotoxin tolerance, a refractory state that develops after a series of endotoxin injections (1). However, considerable evidence has accumulated recently to support the concept that humoral mechanisms play a predominant role. In support of such a concept are the following facts: tolerance can be passively transferred by serum or plasma (2, 3); after RES "blockade" with colloids such as thorium dioxide (Thorotrast), tolerant rabbits still respond with less fever upon challenge with endotoxin when compared to blockaded normals (4) or nonblockaded normals (5); when appropriate endotoxins are employed, cross tolerance is not demonstrable (6). It has been suggested that the humoral factor responsible for tolerance is an antibody (6–9). Nevertheless, it has been recently reported that despite a survey of circulating antibody levels, utilizing five different serological procedures, a satisfactory correlation between endotoxin tolerance and titers of antibody did not exist (10).

The purine antimetabolite 6-mercaptopurine (6-MP) suppresses the immune response to bacterial endotoxins (11, 12). The availability of situations whereby antibody to endotoxin has been suppressed might provide some answers to the question of what role immunologic phenomena are playing in endotoxin tolerance. This report demonstrates that, despite marked suppression of antibody, 6-MP treated rabbits manifest febrile tolerance equal to that of controls. Furthermore, the sera low in antibody passively transfer as much tolerance as the control sera, which contain significantly more antibody. In addition, some of the immunochemical and physicochemical characteristics of the type of antibody elicited are described.

Methods

Rabbits. Over 200 New Zealand albino rabbits of both sexes and weighing approximately 2 kg each were used. Animals were housed and tested in air-conditioned rooms.

Bleedings. All bleedings from marginal ear veins were obtained 24 hours after drug or endotoxin injection or both. The sera were separated by centrifugation and stored at −20° C.

6-Mercaptopurine. 6-MP[1] was prepared immediately before injection by agitating 150 mg of 6-MP monohydrate per ml of 1 N NaOH; this was diluted with sterile pyrogen-free buffered saline [2] to pH 10 to 11 and a concentration of 18 mg per ml. Control animals received equal volumes of 1 N NaOH diluted with buffered saline to pH 10 to 11. Drug-treated rabbits received 18 mg per kg of body weight of 6-MP daily for 7 days beginning simultaneously with endotoxin injections. All injections were into marginal ear veins.

Endotoxin. The endotoxin of *Escherichia coli* 0127 (lot no. 450390) [3] was employed throughout. Concentrated stock solutions of 0.5 or 1 mg per ml were prepared by dissolving the endotoxin in sterile buffered saline and stored at −20° C. Stock solutions were defrosted and diluted with saline so that the desired concentrations were in volumes of 1 ml; these were refrozen and defrosted immediately before administration. Sterile, pyrogen-free glassware, syringes, and needles were used.

Endotoxin tolerance. All rabbits, 6-MP treated and controls, received 7 daily injections of 3 μg of endotoxin, and groups were challenged on day 8 with varying doses of lipopolysaccharide to ascertain the level of tolerance.

Temperature recording. Temperatures (°C) were

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[1] Kindly supplied by Dr. George Hitchings, Burroughs Wellcome & Co., Tuckahoe, N. Y.

[2] Buffered saline at pH 7.4, 0.85% NaCl solution prepared (for 1 L) as follows: M/15 Na_2HPO_4, 84.1 ml; M/15 KH_2PO_4, 15.9 ml; 8.5 g NaCl; 900 ml demineralized water.

[3] Difco Laboratories, Detroit, Mich.

monitored and recorded with thermistors [4] inserted 4 to 6 inches into the rectum and connected to a recording telethermometer.[4] All rabbits were trained for 6 to 8 hours in wooden stocks with loosely fitting collars 1 day before testing. On the day of study, temperatures were monitored for at least 1 hour before injection to insure a steady base line; only rabbits with a base line temperature between 38.5 and 39.9° C were used. Febrile responses were plotted on 1- × 1-inch standard graph paper where 1 hour and 1 degree each equaled 1 inch. The area in square centimeters under a 5-hour fever curve was measured with a compensating polar planimeter [5] and designated as the fever index (FI).

Passive transfer studies. Tolerance was established in groups of 6-MP treated and control rabbits as previously described. On day 8, 24 hours after the seventh daily injection of both endotoxin and 6-MP or control diluent, the rabbits were exsanguinated by cardiac puncture using sterile lightly heparinized syringes. The plasma was separated by centrifugation, pooled, and frozen at −20° C until used. Plasma from normal rabbits was similarly obtained, separated, and stored. Normal recipient rabbits were trained 1 day before passive transfer. Plasma was defrosted and placed in a 37° C water bath for 1 hour on the morning of the transfer. After observing a steady base-line temperature for 1 hour, 10 ml per kg of plasma was infused intravenously into the normal recipients. One to one and one-half hours later, if the recipient rabbits' temperatures remained stable, 0.1 μg of endotoxin was administered intravenously.

Antibody determinations. Bentonite particles were coated with endotoxin, and flocculation of these particles by serial twofold dilutions of serum or fractions after gel filtration was observed (13).

Serum bactericidal activity was assayed as previously described (14) on pooled sera obtained 24 hours after the final (seventh) injection of endotoxin and drug. Antibody activity was expressed as the reciprocal of the serum dilution calculated to kill 50% of the inoculum of *E. coli* (SD_{50}).

Immunoelectrophoresis. Pre- and post-treatment sera were studied by immunoelectrophoresis (15), as modified for microscope slides (16). Goat antirabbit serum was used to develop immunoelectrophoresis patterns. Absorption of goat antirabbit serum was carried out with purified rabbit gamma globulin [6] obtained by DEAE cellulose chromatography as follows: 1 mg of gamma globulin was mixed with the antiserum and centrifuged; the supernate was then used to develop the immunoelectrophoresis pattern in which the characteristic gamma globulin precipitin arc was no longer visible.

Gel filtration with Sephadex G 200. A column 117 cm in length and 2.2 cm in diameter was packed by gravity flow with Sephadex G 200, which had been repeatedly washed with 0.1 M sodium chloride (buffered with 10% by volume of borate buffer pH 8.0). Pooled sera (2.75 to 5.5 ml) from both 6-MP–treated (three animals) and control rabbits (three animals) were applied to the column, and the fractionation was carried out with borate-buffered 0.1 M sodium chloride (pH 8.0). The flow rate was 7 to 8 ml per hour, and fractions were collected at a rate of 10 minutes per tube. The protein concentrations of the fractions were measured by absorption at 280 mμ in a Beckman DU spectrophotometer. Fractions were stored at 4° C for 3 to 7 days before determination of their antibody content.

Concentration of fractions after gel filtration. Fractions from the central portion of the three peaks after gel filtration were pooled and designated Fractions I, II, and III, respectively. These fractions were concentrated by centrifugation in a Spinco model L ultracentrifuge for 18 to 20 hours at 39,000 rpm in a no. 40 angle rotor. The upper supernates were removed with a Pasteur pipette, and the remaining 1 to 2 ml of supernate and centrifugate were mixed. Protein concentration of these mixtures was determined with the biuret method (17). Immunoelectrophoresis and antibody determinations were done on samples of each. Portions of each fraction were then diluted with saline so that the protein concentration of each of the three fractions of both groups of eluates was equal.

Analytical ultracentrifugation. Pre- and post-treatment sera from a 6-MP endotoxin-treated rabbit as well as a control endotoxin-treated rabbit were diluted 1:4 with saline and analyzed in a Spinco model E ultracentrifuge. The control sera were run at 17.3° C and the 6-MP sera at 19.4° C. The patterns on the resultant plates were projected onto graph paper and traced. The areas under

TABLE I

Effect of 6-mercaptopurine (6-MP) on antibody response to endotoxin

Treatment	Number*	Titer†
6-MP	78	1:2.1 (1.6–2.7)
Control	81	1:40.0 (31.0–51.5)

* Bleedings obtained 24 hours after the seventh daily injection of endotoxin and 6-MP or control solution.

† Geometric mean titers with 95% confidence intervals as determined by bentonite flocculation.

TABLE II

Bactericidal antibody response to Escherichia coli endotoxin: effect of 6-MP

Treatment	Number*	Titer†
6-MP	16	1:5,654 (1:4,900–1:6,200)
Control	15	1:35,450 (1:33,800–1:39,000)

* Bleedings obtained 24 hours after the seventh daily injection of endotoxin and 6-MP or control solution. Sera pooled.

† Geometric mean SD_{50} (serum dilution calculated to kill 50% of the inoculum of *E. coli*) value with total range for three pools obtained on separate occasions.

[4] Yellow Springs Instrument Co., Yellow Springs, Ohio.

[5] Keuffel and Esser, Hoboken, N. J.

[6] Courtesy of Dr. Sheldon Dray.

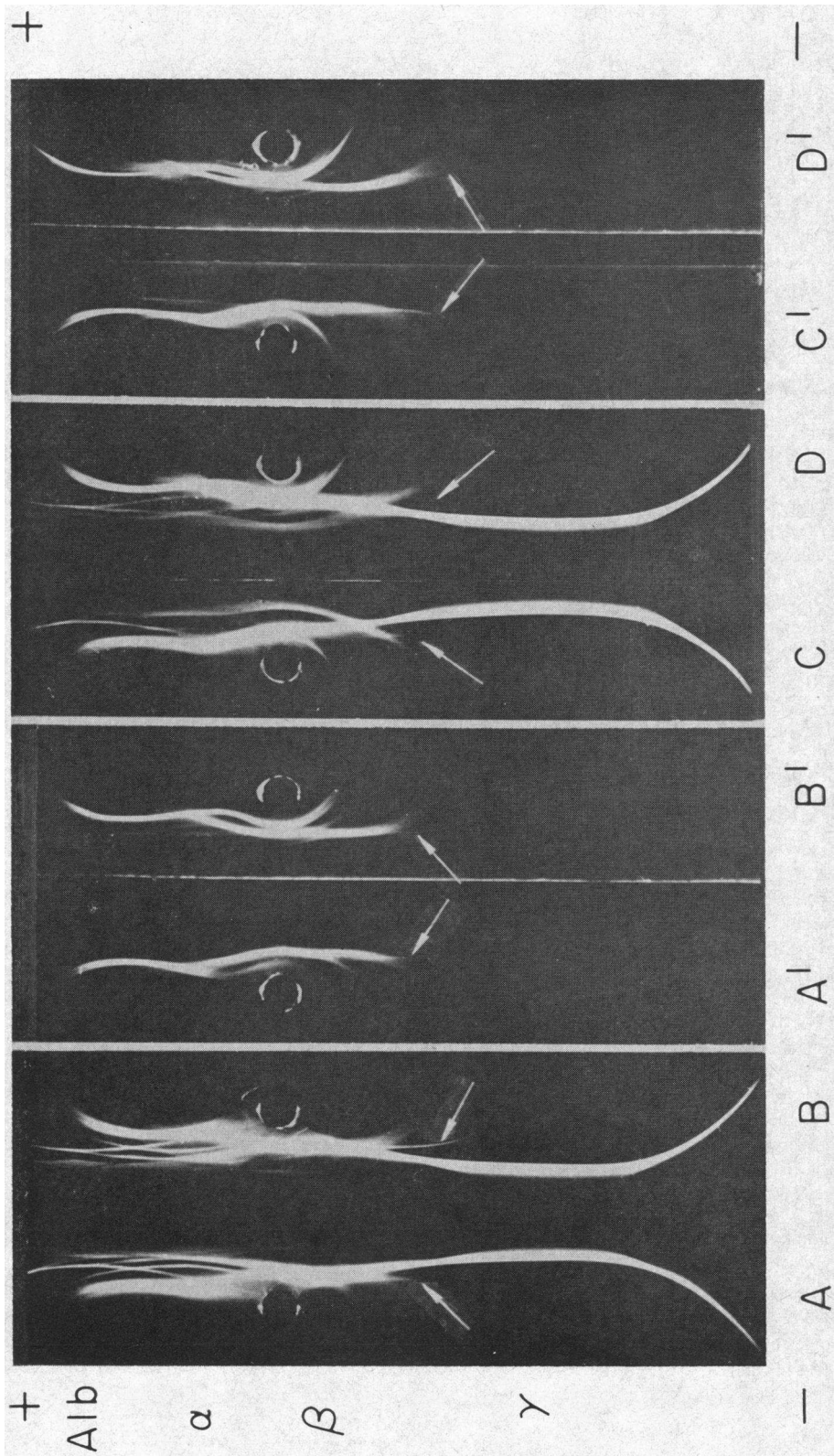

FIG. 1. IMMUNOELECTROPHORESES OF PAIRED SERA FROM 6-MERCAPTOPURINE (6-MP)-TREATED AND CONTROL ENDOTOXIN TOLERANT RABBITS. Serum A is before endotoxin, and B is from the same animal after 7 days of endotoxin and the control diluent solution. A' and B' are the same sera subjected to electrophoresis after the antiserum had been absorbed with gamma globulin to prevent development of the gamma globulin precipitin arc. Note increased thickness of macroglobulin precipitin arc after development of tolerance (arrows). Serum C is before endotoxin, and D is from the same animal after 7 days of endotoxin and 6-MP. C' and D' are the same sera subjected to electrophoresis after the antisera had been adsorbed with gamma globulin to remove the gamma globulin precipitin arc. In contrast to the control sera note that the macroglobulin precipitin arc did not thicken (arrows). + = anode. − = cathode. A goat antirabbit sera was used throughout.

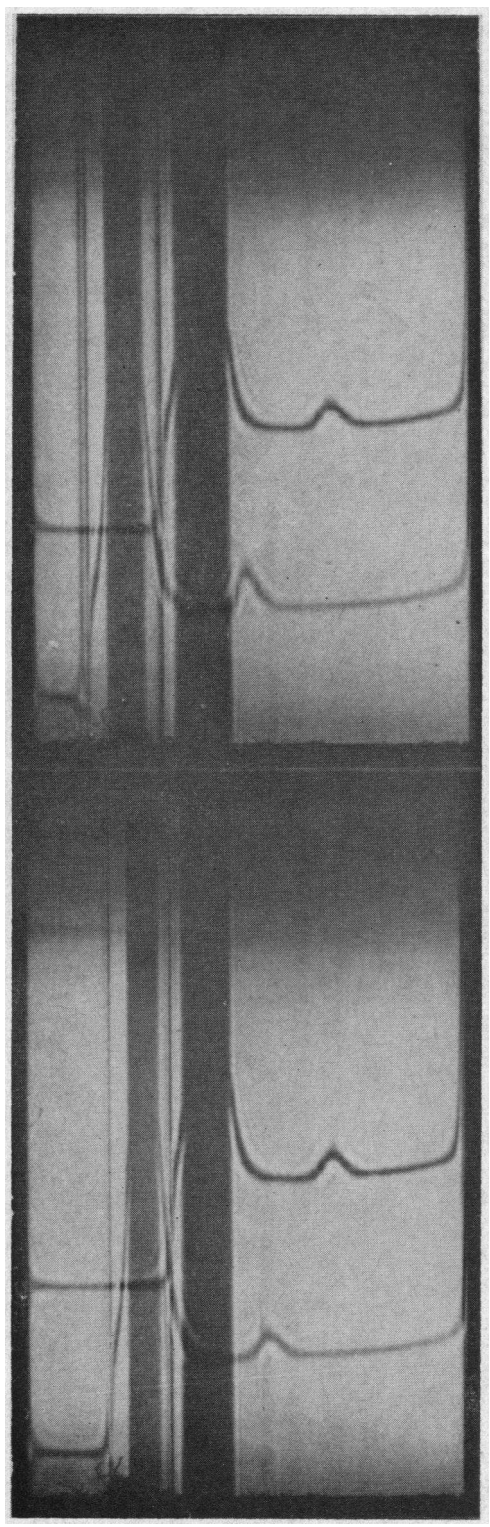

FIG. 2. ANALYTICAL ULTRACENTRIFUGATION OF PAIRED SERA FROM 6-MP–TREATED AND CONTROL ENDOTOXIN TOLERANT RABBITS. Upper 2 patterns are the 19 S macroglobulin patterns from before (uppermost) and after the induction of endotoxin tolerance (control). Planimetry of these revealed a concentration of 2.2 mg per ml before and 3.5 mg per ml after the establishment of tolerance. Lower 2 patterns are before and after (lowest pattern) the establishment of endotoxin tolerance plus 6-MP. Both sera had a concentration of 2.2 mg per ml of 19 S macroglobulin. Picture was taken after 20 minutes at 59,780 rpm with a bar angle of 60°.

the curves were measured with a planimeter, and the concentration of the proteins was calculated by correcting for radial dilution and assuming $dn/dc = 1.88 \times 10^{-4}$ (4 g) (18).

Results

Suppression of antibody. After the daily injections of both antigen and drug there was significant suppression of antibody in the 6-MP–treated group ($p < 0.01$, t test) (Table I). Antibody to endotoxins has only been rarely detected in untreated N.I.H. rabbits by the bentonite flocculation technique; therefore, such studies are not reported. Bactericidal assays done on three groups of pooled sera revealed that levels of bactericidal antibody had been similarly suppressed (Table II). Normal rabbits from this colony have base-line bactericidal assay titers between 1:80 and 1:500.

Immunoelectrophoretic studies. Immunoelectrophoresis is not a quantitative technique. However, if care is taken to assure similarities of technique and quantities of sera applied, then a semiquantitative estimate of gross changes may be made. The antisera employed demonstrated a precipitin arc in the fast gamma area, which was cysteine-HCL sensitive and present in the first peak from a Sephadex G-200 column (19); thus, this protein has the properties of a macroglobulin. It has recently been shown that the antibody to endotoxin is mainly of the macroglobulin type (20, 21). As illustrated in Figure 1, this macroglobulin line thickens after endotoxin administration, but when the animal also receives 6-MP the line either fails to thicken or disappears at the dilutions employed (Figure 1). These studies have been performed on many occasions with a large number of sera, and the results always demonstrated the thickening of the macroglobulin line from the tolerant control sera and either a diminution or no change in the macroglobulin line from the 6-MP–treated animals. When 7 S gamma globulin is added to the antisera so that no gamma globulin

TABLE III

Antibody levels of fractions after gel filtration on Sephadex G-200

Sera	Fraction I	Fraction II	Fraction III
	Protein concentration as eluted from column		
6-MP*	1:1†	Negative	Negative
Control*	1:64	1:8	Negative
	Diluted to equal protein concentration		
6-MP	1:1	Negative	Negative
Control	1:64	1:2	Negative

* Pooled sera from three rabbits after 7 days of endotoxin and 6-MP or control solution.
† Bentonite flocculation titers.

precipitin line appears, these alterations of macroglobulin are readily demonstrable (Figure 1).

Analytical ultracentrifugal studies. Analytical ultracentrifugation was performed using the sera of animals both before and after endotoxin and 6-MP or the diluent. Since the temperatures of the runs were less than 20° C (between 17° and 19° C) and the concentration of the protein was high, the sedimentation coefficients were found to be about 16 S (15.7 to 16.6) and 5.7 S (5.5 to 5.9); if run at 20° C and infinite dilution, the sedimentation coefficients would have been closer to the expected 19 S and 6.7 S, respectively. The concentration of the macroglobulin (19 S) protein was 2.2 mg per ml before and 3.5 mg per ml after endotoxin in the control animal. On the other hand, the animal that received 6-MP in addition to endotoxin also had a pretreatment level of 2.2 mg per ml; however, after administration of both agents, the level did not increase and remained at 2.2 mg per ml (Figure 2). After endotoxin, the control animals had 9.3 mg per ml of 7 S gamma globulin and the 6-MP–treated animal had, in contrast, 2.3 mg per ml.

Gel filtration. After gel filtration on Sephadex G-200 only the effluent from the first fraction of the 6-MP–treated sera had any antibody titer at all, and this was only weakly positive when undiluted (Table III). The first fraction from the non-6-MP–treated animals, however, had a titer of 1:64, and the second was positive at 1:8. The latter titer probably represents some overflow of macroglobulin. To further define whether these data were merely due to the difference in protein content of the fractions or due to actual suppression of antibody production, the effluents of the three fractions from both non-6-MP– and 6-MP–treated sera were diluted so that the protein concentrations of all six were equal to that of the first fraction from the 6-MP–treated group since it was lowest in protein content. After these dilutions the first fraction of the 6-MP-treated sera was still only weakly positive when undiluted. The control sera still had a titer of 1:64 in the first fraction, but the titer of the second had decreased to 1:2 (Table III). When the effluents from the first fractions were subjected to immunoelectrophoresis, the macroglobulin precipitin line was obvious only in the first fraction from the control animals but not in that of the 6-MP–treated animals with the conditions employed.[7]

Effect of 6-MP on the febrile response to endotoxin. To assure that 6-MP alone for 7 days would not alter the febrile reactivity of rabbits the following experiments were done. Fifteen rabbits received 18 mg per kg of 6-MP daily for 7 days, and 16 rabbits were given the NaOH-saline control solution. Both groups were challenged with 0.5 μg of endotoxin on day 8. The 6-MP–treated and control groups had mean fever index ± standard errors of 32.5 ± 2.8 and 37.2 cm^2 ± 3.0, respectively, which were not significantly different (*t* test).

After daily injections of 3 μg of endotoxin and either 6-MP or the control solution, groups of rabbits were challenged with varying quantities of endotoxin. The results of these experiments are

TABLE IV

Effect of 6-MP on febrile tolerance to endotoxin

Endotoxin dose*	Fever indexes†	
	6-MP	Control
μg	cm^2	
3	18.2 ± 1.3 (16)‡	21.0 ± 3.1 (16)
30	47.3 ± 3.1 (15)	43.0 ± 2.3 (25)
150	55.9 ± 3.6 (13)	54.1 ± 3.1 (12)

* Dose administered on the day following 7 daily injections of 3 μg of *E. coli* endotoxin and 6-MP or control solution.
† Data expressed as mean fever indexes ± standard error.
‡ Number in parentheses = number of animals.

[7] The gel filtration studies have been repeated utilizing pooled sera from both 6-MP–treated and control tolerant rabbits, and the results confirmed the findings noted above.

given in Table IV. Despite the significant differences in antibody (Table I), the febrile responses at each challenge dose are strikingly similar and not significantly different (t test), indicating that febrile tolerance was not affected by either the administration of 6-MP or the levels of circulating antibody as measured in this study.

Passive transfer studies. To further define the effect of 6-MP and the suppression of antibody on tolerance, passive transfer studies were performed (Table V). Both the 6-MP–treated (13 donors) and control (30 donors) tolerant plasma passively transferred significant tolerance to the recipient animals ($p < 0.01$, t test) when compared to normal plasma. However, the tolerance passively transferred by the plasma of the 6-MP–treated tolerant rabbits was not significantly different from that of the control tolerant plasma. The fever curves of the recipient animals, which received plasma from either 6-MP or control tolerant animals, were consistently of the monophasic type, whereas the responses of the control animals were uniformly biphasic.

TABLE V

Effect of 6-MP on passive transfer of endotoxin tolerance

Donor plasma	Number of recipients	Fever indexes
		cm^2
Normal	13	33.5 ± 2.6*
6-MP tolerant†	8	18.6 ± 1.5
Control tolerant†	11	17.9 ± 1.7

* Data expressed as mean fever indexes ± standard error.

† Plasma obtained after 7 days of endotoxin and 6-MP or control solution.

Discussion

Recently, it has been suggested that antibody plays the predominant role in endotoxin tolerance (6–9), although for years hyperfunction of the RES was considered the major mechanism for its development. Successful passive transfer of endotoxin tolerance has been the major supporting evidence for the part played by antibody, although very few actual antibody assays have been reported. On the other hand, it was recently noted that, despite a survey of five techniques for measuring circulating antibody, a definite correlation between levels of antibody and tolerance could not be established (10). The present study lends further support to this lack of correlation. There can be little doubt that a humoral factor is responsible for the passive transfer of tolerance; however, this factor is not necessarily antibody. It has recently been reported that fractions of sera from tolerant rabbits containing macroglobulin are capable of passively transferring tolerance (22, 23). These preliminary findings were interpreted as demonstrating that an "antibody" of the 19 S class was responsible for tolerance, although no information on antibody determinations was provided. In the present study the level of 19 S macroglobulin did not increase in the 6-MP–treated animal and was only a little more than one-half as high as the macroglobulin level in the control animal. Despite these differences, passive transfer of an equal degree of tolerance was accomplished with plasma from both the 6-MP–treated and control animals.

It is possible that the low levels of antibody present in the sera of the 6-MP–treated animals may be sufficient to provide maximal tolerance, thus resulting in the described results. Such a hypothesis, however, would seem unlikely, since tolerance was present even when the animals were challenged with a wide range of endotoxin dosages in an attempt to elicit quantitative differences in the two groups. It is also possible that an antibody, not measurable with presently available techniques, is responsible for endotoxin tolerance. It may be that the RES and antibody in addition to other as yet unidentified factors are all contributing to the development and maintenance of this refractory state. As we have recently pointed out, antibody probably plays some role (10), but the elucidation of its actual contribution awaits further study.

The significant suppression by 6-MP of circulating antibody after seven daily injections of endotoxin confirms and extends initial observations utilizing a single injection of *Salmonella typhosa* endotoxin (12) and up to 10 daily injections of the endotoxins of *Salmonella enteritidis* and *E. coli* (11). In addition, this report notes for the first time that these agents are capable of suppressing the formation of bactericidal antibody as well. Whether the antibody response to bacterial endotoxins is of the primary or secondary type is

still not clear. However, the presence of these antibodies (titers of 1:80 to 1:500 by bactericidal assay) in the sera of rabbits before overt antigenic stimulation suggests that the response is more likely of the secondary type. Suppression by 6-MP of the secondary antibody response to protein antigens has been previously reported, but only after doses in the range of those used in the present studies were employed (24).

Since the original investigation of Schwartz, Stack, and Dameshek, the purine antimetabolites have become very useful tools in the study of many immunologic problems (25, 26). When protein antigens are employed as primary antigenic stimuli, 7 S but not the 19 S antibody is suppressed by 6-MP (27); however, doses of 6-MP (10 mg per kg) were employed that were smaller than those used in the present studies. The demonstration that 19 S macroglobulin and antibody to endotoxin does not increase, as expected, after 6-MP treatment suggests that the effects elicited by the use of this antimetabolite may depend upon the antigen employed, the type immune response elicited, or the dose of antimetabolite administered. It has been previously reported that 6-MP will suppress gamma globulin levels in normal rabbits (i.e., no antigen was administered) (28). In the present work, evidence for suppression of 19 S macroglobulin levels has been presented. The gel filtration studies demonstrate that the formation of specific antibody is likewise depressed. Thus, the immunosuppressive capability of 6-MP may be related to its ability to selectively inhibit the formation of antibody and the immunoglobulins rather than to nonspecific depression of protein synthesis.

Summary

Endotoxin tolerance was induced in rabbits by 7 daily intravenous injections of *Escherichia coli* endotoxin. Half of the animals also received 6-mercaptopurine, whereas the rest were given a control solution. Antibody response of the 6-mercaptopurine (6-MP)–treated animals was significantly suppressed. Furthermore, 19 S macroglobulin levels were also lower in the drug-treated group. Despite the differences in antibody levels, 6-MP–treated animals, when given a range of challenge doses of endotoxin, were as tolerant as control animals. Similarly, "antibody poor" plasma (i.e., from 6-MP–treated animals) passively transferred febrile tolerance as well as did "antibody rich" (control) plasma. These findings are not consistent with circulating antibody being the sole, or even major, factor responsible for endotoxin tolerance.

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