EFFECT OF 6-MERCAPTOPURINE (6-MP) ON DIFFERENT CLASSES OF ANTIBODY*

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(Received for publication, April 9, 1965)

Many antigens have been found to induce the formation of several molecular classes of antibodies. Under most circumstances, the first antibody to be synthesized in response to antigenic stimulation is a 19S (γM) globulin, while 7S immunoglobulins (γG) appear somewhat later. The factors controlling the synthesis of these 2 proteins are poorly understood. The recent report of the selective suppression of γG-antibodies in rabbits by 6-mercaptopurine (6-MP) suggests that the formation of each of these immunoglobulins is controlled by a separate mechanism (1). Furthermore, 19S antibody synthesis in man appears more resistant to 6-MP treatment than 7S antibody formation (2, 3). In the present paper the effect on γM- and γG-antibody formation of 6-MP has been further investigated in the primary and secondary response of mice and rabbits.

Materials and Methods

Animals.—White Swiss mice of both sexes weighing 25 to 30 gm and white outbred male or female rabbits weighing 3.5 to 4.5 kg were used.

Immunization.—Rat erythrocytes were obtained by cardiac puncture from Wistar albino rats and washed 3 times with buffered (pH 7) saline. Twenty groups of 10 mice were immunized with an intravenous dose of 0.25 ml of a 50 per cent suspension of rat erythrocytes (large dose). Sixteen groups of 10 mice were immunized with an intravenous injection of a 2.5 per cent suspension of rat erythrocytes (small dose). A second injection of the same dose of rat erythrocytes for the appropriate group was given either 2 or 3 weeks later in order to study the anamnestic response. Twelve rabbits were injected intravenously with 1 ml of a 50 per cent saline suspension of human type A erythrocytes washed 3 times. A similar injection was given 20 days later.

Administration of 6-MP.—Six-mercaptopurine (6-MP)[1] was brought into solution in distilled water with the minimal amount of NaOH necessary to dissolve the drug. Eight groups of 10 mice were treated daily with 50 mg/kg/body weight intraperitoneally for the first 10 days of the immunization. Ten groups of mice were given 150 mg/kg/body weight intraperitoneally for 4 days beginning on the day of the second injection. Six rabbits received two 10-day courses of 6-MP (6 mg/kg) beginning on the day of each antigen injection.

* This work was supported by grants No. A-3777 and A-4819 of the United States Public Health Services.

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[1] Kindly given to us by Burroughs, Wellcome and Co., Inc., Tuckahoe, New York.

Serological Tests.—The mice were bled from a retroorbital vein with a 0.2 ml calibrated pipette. In order to obtain enough serum for serologic studies, the blood samples from each group of 10 mice were pooled and allowed to clot. Bleedings were made at regular intervals after each antigen injection.

The rabbits were bled from a marginal ear vein before the injection of the antigen and at 5-day intervals thereafter.

Treatment of the Serum with 2-Mercaptoethanol (ME).—Pooled mouse serum or rabbit serum was diluted in an equal amount of buffered saline and incubated with ME (in a final concentration of 0.1 M) for 2 hours at room temperature. After incubation the sera were dialyzed in the cold overnight against buffered saline containing 0.02 M iodoacetamide.

Antibody Activity.—Hemagglutination tests were performed with a 2 per cent suspension of rat erythrocytes. The mouse sera were diluted with buffered saline containing 2 per cent normal mouse serum. The rabbit sera were diluted in 0.15 M saline. The tubes were incubated in a water-bath at 37°C for 30 minutes. The hemagglutination was read microscopically.

Sucrose Density Gradient Ultracentrifugation.—The method described by Edelman, Kunkel, and Franklin was used (4). Pooled mouse serum (0.3 ml) was layered over a gradient formed

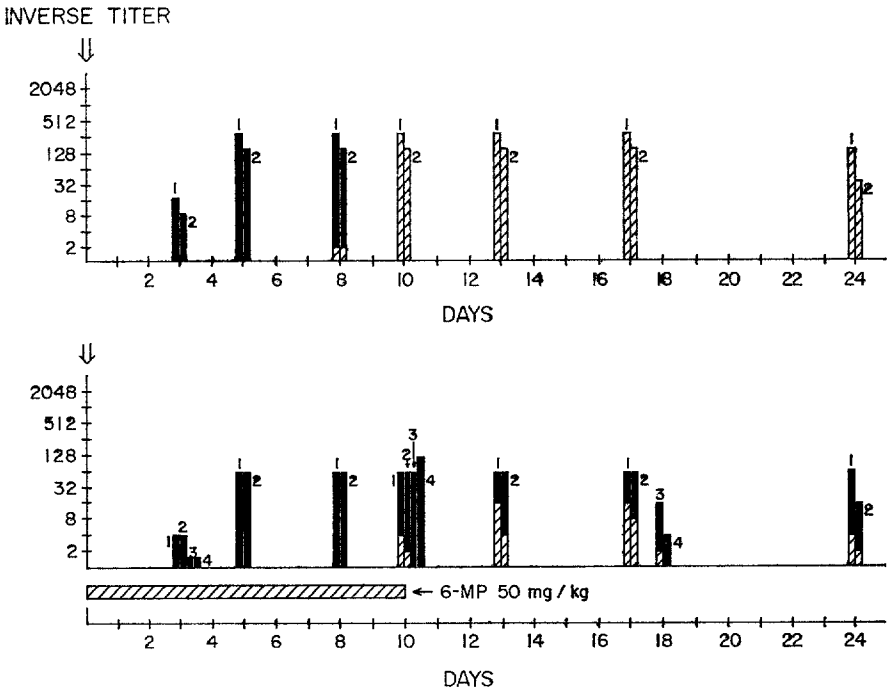

FIG. 1. Effect of subsupressive dose of 6-MP on the primary response in mice. At day 0, the animals were immunized intravenously with 0.25 ml of 50 per cent suspension of rat erythrocytes. Six representative groups are shown in this figure. Each column represents the result of hemagglutination obtained with pooled sera from a group of 10 mice. Two groups served as control and 4 groups were treated with 6-MP. Black, column: ME-sensitive antibody activity. Hatched column: ME-resistant antibody activity.

from 37.5, 25, and 12.5 per cent sucrose. Centrifugation was performed in a Spinco model L ultracentrifuge with a SW 39 swinging bucket rotator at 32,000 RPM for 16 to 18 hours. Twelve to 14 fractions were collected through a small perforation at the bottom of the centrifuge tube. After dialysis against 0.15 M saline, the protein concentration of the fraction was determined by the Folin-Ciocalteu method (5). In some experiments fractions were pooled to obtain a rapidly sedimenting fraction rich in 19S globulin and essentially free of 7S globulin molecules, and a fraction containing the bulk of the 7S globulin. In other experiments individual fractions were examined serologically. Aliquots of each individual or pooled fraction were then diluted 1:2 with buffered saline and treated with ME. Hemagglutinin activity was determined in each non-treated and ME-treated sample. Sera from control and 6-MP–treated mice were usually obtained on the same day and examined simultaneously. In 1 sucrose gradient the same pooled serum from 6-MP–treated animals was run together with the ME-treated serum. In another experiment a human macroglobulin (19S) was used as a marker.

Chromatography on sephadex G-200.—Pooled mouse sera (0.9 to 1 ml) were applied to columns (2.0 × 90 cm) of sephadex G-200 and eluted in the cold at a rate of 4 to 9 ml per hour with 0.5 M NaCl. Four ml fractions were collected. Protein concentration was measured at 280 mμ. The fractions corresponding to either the 19S or 7S peak were then dialyzed overnight against buffered saline and concentrated by vacuum dialysis. An aliquot of the pooled fraction was treated with mercaptoethanol and hemagglutinin activity was determined in the untreated and ME-treated pools. In two experiments the intermediate fractions between the 19S and 7S peak were also concentrated and treated the same way. A protein determination of the pooled fractions was performed and the titer of antibody activity in all fractions was expressed in terms of its protein concentration.

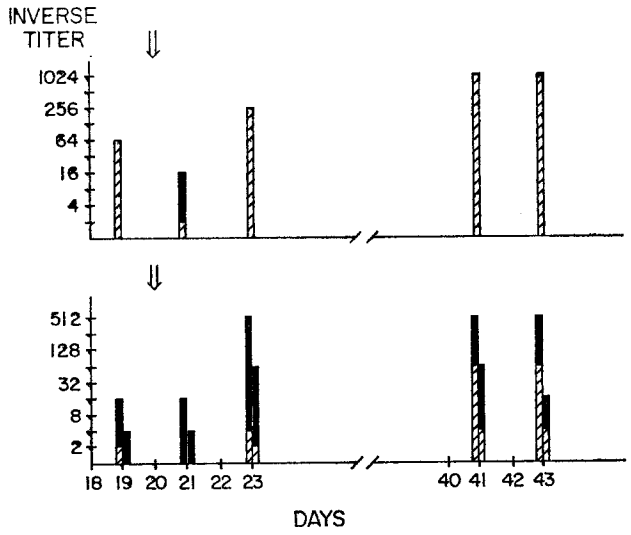

FIG. 2. Effect on the anamnestic response of 6-MP given during the primary response. Each animal was injected intravenously with 0.25 ml of 50 per cent suspension of rat erythrocytes at day 0 and a second similar injection was given at day 20. The results are represented as in Fig. 1. Black column: ME-sensitive antibody activity. Hatched column: ME-resistant antibody activity.

RESULTS

Effect of 6-MP on the Primary Immune Response.—Two-mercaptoethanol sensitive antibody was first detected in all 12 groups of mice 3 days after an injection of 0.25 ml of a 50 per cent suspension of rat erythrocytes. The maximum titer of this antibody was reached on the fifth day and 10 days after

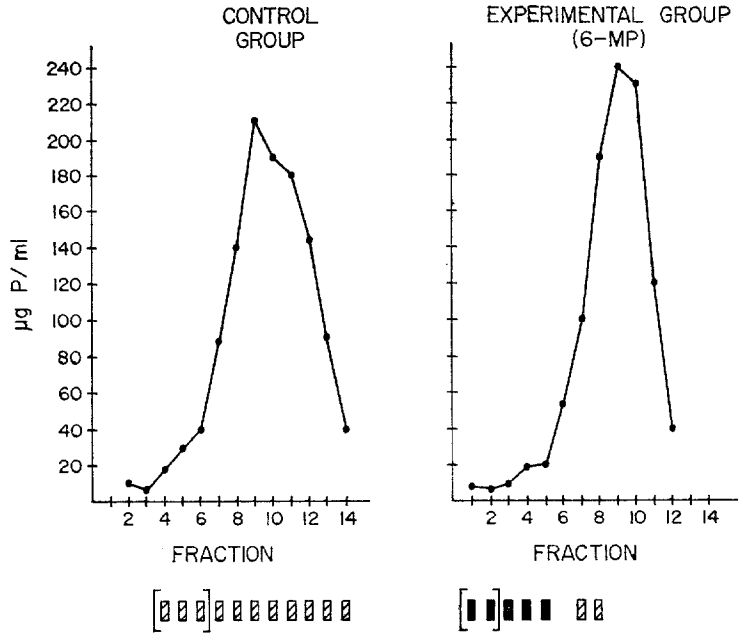

FIG. 3. Sucrose gradient ultracentrifugation of pooled sera from control group (left panel) and experimental group (right panel) treated with 6-MP during the first 10 days of immunization. All animals were immunized at day 0, reinjected at day 20 and bled at day 43. The protein content of the fractions is indicated in μg of protein per ml. The hemagglutinin activity of each fraction is represented below the abscissa. Brackets represent weak antibody activity. Black column: ME-sensitive antibody activity. Hatched column: ME-resistant antibody activity.

antigen administration it was no longer found in the serum. During the next 14 days all the antibody was ME-resistant (Fig. 1). Six-mercaptopurine administration did not prolong the induction period; however, the 4 groups of mice given 6-MP had lower titers of antibody initially. The appearance of ME-resistant antibody was delayed by 6-MP in 2 groups of mice and diminished in all 4 groups throughout the experiment. In contrast to the control groups, the 6-MP–treated mice continued to produce ME-sensitive antibody for at least 24 days after the antigenic stimulus. Sera of 6-MP–treated animals obtained 19 days after immunization were analyzed by sucrose gradient ultra-

centrifugation. At the same time sera of control mice bled at day 5, which contained only ME-sensitive antibody activity, were similarly analyzed. In both cases the ME-sensitive antibody was found only in the heavy fractions. The ME-resistant antibody present in the sera of the 6-MP–treated mice was found in the upper fractions.[2]

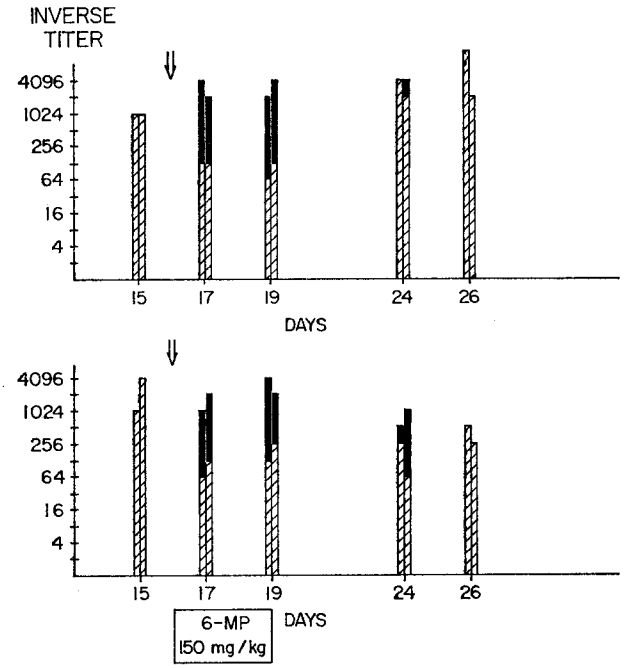

FIG. 4. Effect on the anamnestic response of 6-MP given during the anamnestic response, "large dose" of rat erythrocytes. Each animal was immunized at day 0 with 0.25 ml of 50 per cent suspension of rat erythrocytes. At day 15 only ME-resistant antibody activity could be demonstrated. After a second intravenous injection of 0.25 ml of 50 per cent suspension of rat erythrocytes at day 16, a considerable proportion of the antibody activity became ME-sensitive. Six-mercaptopurine administration did not influence the pattern of the antibody response.

The results in control and 6-MP–treated mice given 0.25 ml of 2.5 per cent suspension of rat erythrocytes were the same as those given the "large dose" (0.25 ml of 50 per cent suspension) and will not be described in detail.

Effect on the Anamnestic Response of 6-MP Given during the Primary Response.—One group of 10 control mice and 2 groups of 10 experimental mice were reinjected on day 20 with 0.25 ml of a 50 per cent suspension of rat erythrocytes. The results are presented in Fig. 2. Two-mercaptoethanol–sensitive agglutinin was found in the sera of control mice only on the 1st day after the

[2] Sucrose gradient was made up with 15 M NaCl and distilled water. No difference was seen.

booster injection; subsequently all the antibody was ME-resistant. In contrast, ME-sensitive antibody was present in the 6-MP–treated mice for at least 43 days after the second dose of antigen. The agglutinin titers in all groups of

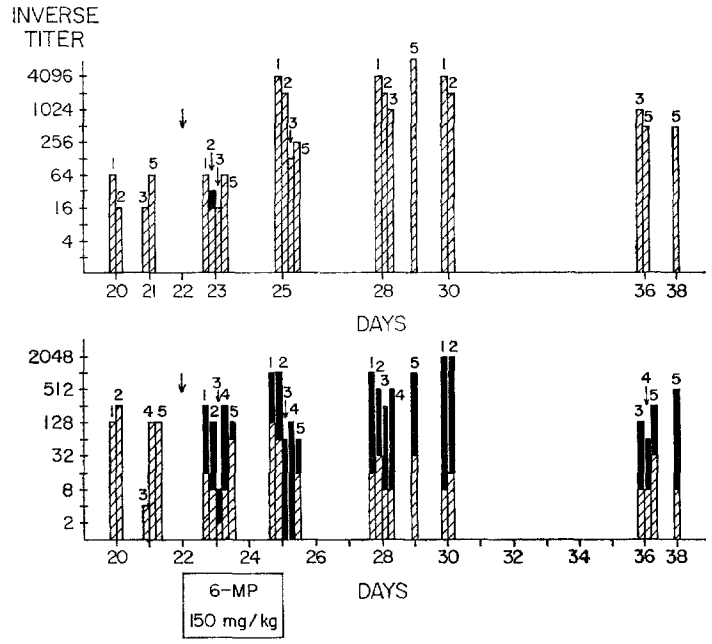

FIG. 5. Effect on the anamnestic response of 6-MP given during the anamnestic response, "small dose" of rat erythrocytes. Each animal was immunized at day 0 with 0.25 ml of 2.5 per cent suspension of rat erythrocytes. At day 20 and 21 all animals exhibited ME-resistant hemagglutinin activity. After a second injection with the same dose, only 1 of 5 control groups exhibited a small proportion of ME-sensitive antibody activity (group 2). In contrast, all 5 groups of animals which received 6-MP developed a large proportion of ME-sensitive antibody activity. Note the drop of ME-resistant hemagglutinin activity in the 6-MP–treated mice The 5 groups of control and the 5 groups of 6-MP–treated mice are numbered consistently throughout the experiment with numbers 1 to 5. Black column: ME-sensitive antibody activity. Hatched column: ME-resistant antibody activity.

mice were similar. A sucrose density gradient analysis done 23 days after the booster injection is shown in Fig. 3.

Effect on the Anamnestic Response of 6-MP Given during the Anamnestic Response.—Eight groups of 10 mice immunized with a large dose of rat erythrocytes and reinjected on either day 14 or 20 with the same dose of antigen produced ME-sensitive in addition to ME-resistant antibody for only a few days after the second antigen injection. Six-mercaptopurine treatment (150 mg/kg) for 4 days starting on the day of the second antigen injection did not alter the immune response. Four representative groups are shown on Fig. 4.

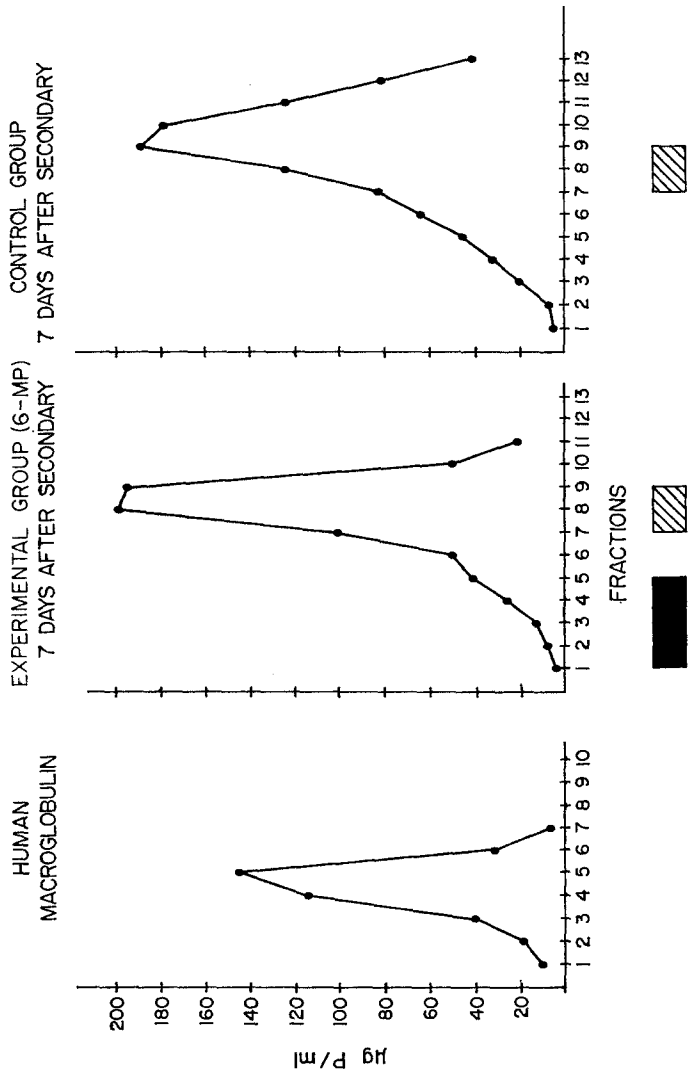

FIG. 6. Sucrose gradient ultracentrifugation of control group 5 and experimental group 5 of series shown in Fig. 5. Pooled sera of mice bled at day 29 (7 days after second antigen injection) have been used for this experiment. At the same time a purified human γ M-globulin was assayed for comparison. Serum from the experimental group 5 showed hemagglutinin activity in the pooled fractions 1 to 5 which was ME-sensitive. These fractions correspond to the bulk of the human macroglobulin. No heavy molecular antibody activity was demonstrated in the control group. Both sera exhibited light molecular antibody activity which resisted ME treatment.

Five groups of 10 mice injected with a small dose of foreign red cells and reinjected on day 20 with the same dose of antigen produced an anamnestic response consisting almost exclusively of ME-resistant antibody activity. The anamnestic response in 5 groups of mice treated with 6-MP as mentioned above

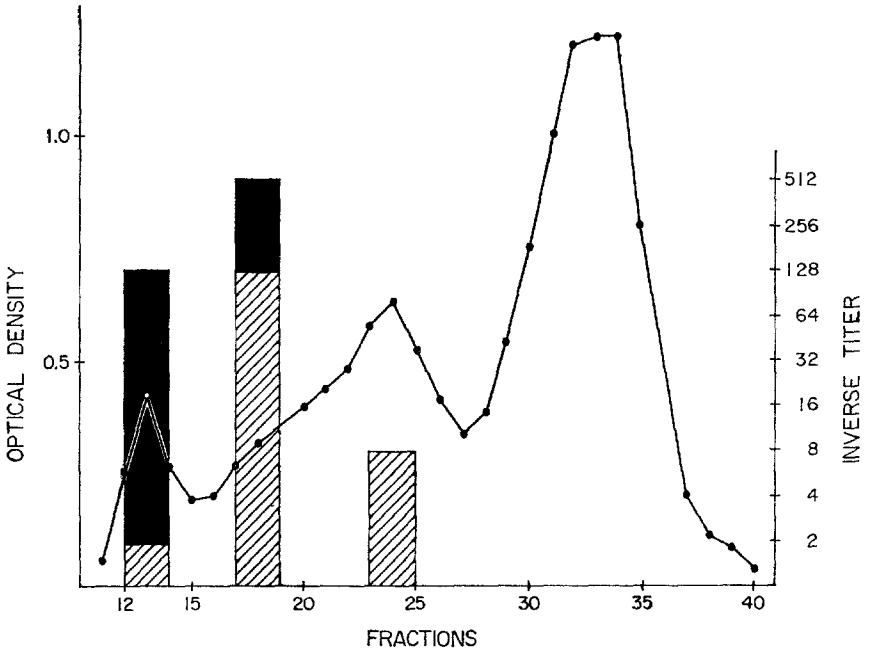

FIG. 7. Chromatography on sephadex G-200. The same pooled serum of experimental group 5, which was submitted to sucrose gradient ultracentrifugation (see Fig. 6) was subjected to chromatography on sephadex G-200. The following 3 pooled fractions were adjusted with buffered saline to a protein concentration of 0.4 mg of protein/ml: fractions 12 to 14, 17 to 19, 23 to 25. These 3 pooled fractions were assayed for hemaglutinin activity. It should be noted that a control serum which exhibited only ME-resistant hemagglutinin activity also showed a ME-resistant titer of 1:2 in the fractions 12 to 14 containing the bulk of γ M-globulin. Black column: ME-sensitive antibody activity. Hatched column: ME-resistant antibody activity.

was qualitatively different. A considerable proportion of antibody activity was ME-sensitive 1 day after the booster injection. Three days later group 3 and group 4 (see Fig. 5) had only ME-sensitive agglutinins. After discontinuation of 6-MP treatment the ME-sensitive antibody in the 3 other groups increased. A large proportion of the antibody activity remained ME-sensitive in all experimental groups for 16 days after the second antigen injection. Untreated and ME-treated serum of the group 1 of 6-MP–treated mice at day 28 were analyzed by sucrose gradient ultracentrifugation. The untreated serum contained antibody activity in the light and heavy fractions, whereas the ME-

treated serum contained antibody activity only in the upper fractions. An aliquot of the same pooled sera was subjected to fractionation on sephadex

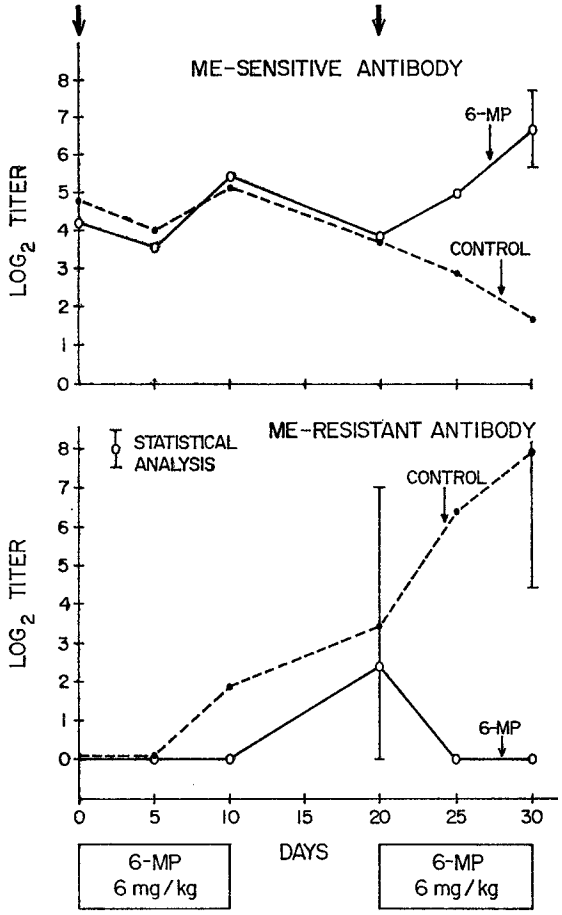

FIG. 8. Effect of subsuppressive dose of 6-MP in the "primary" and "secondary" responses of rabbits injected with human red blood cells. Six control rabbits and 6 animals receiving 6-MP were injected with 1 ml of a 50 per cent suspension of human erythrocytes at day 0 and day 20. Antibody activity (average of titers) is expressed in terms of ME-sensitive (upper panel) and ME-resistant (lower panel) hemagglutinin activity. These results have been analyzed statistically according to the t test of Student.

G-200. The ME-sensitive antibody activity was localized to the fractions corresponding to the 19S globulins and the ME-resistant antibody activity was in the fractions corresponding to 7S globulin. Seven days after the second antigen injection, control and experimental pooled sera were analyzed on sucrose gradient. The result is in Fig. 6. An aliquot of serum of 6-MP group number 5 was

also subjected to chromatography on sephadex G-200 (Fig. 7). These 2 experiments confirmed that most of the ME-sensitive antibody activity of serum of this group was found in the heavy fractions on sucrose gradient, and in the fractions corresponding to 19S globulins on sephadex G-200.

Experiments in Rabbits.—All 12 rabbits used in this experiment had naturally occurring ME-sensitive heterophile antibody against human type A red cells. The injection of 1 ml of 50 per cent saline suspension of human type A red cells did not modify the titer of this ME-sensitive antibody in either control or experimental animals. Immunization resulted in the formation of ME-resistant antibody in the 6 control animals. The titer of these antibodies increased on day 20. A 10-day course of 6-MP treatment beginning on the day of antigen injection resulted in a delayed appearance of ME-resistant antibody. Three of 5 animals formed ME-resistant antibody only after the drug was discontinued at a lower titer than the control animals. In the control group a second similar antigen injection at day 20 resulted in an increased titer of ME-resistant antibody while the ME-sensitive antibody titer decreased. A second 10-day course of 6-MP beginning on the day of the second antigen injection suppressed the production of the ME-resistant antibody response, but not that of the ME-sensitive antibody response (Fig. 8).

DISCUSSION

The mouse and rabbit antibodies studied in these experiments were analyzed by 3 methods: differential susceptibility to 2-mercaptoethanol, sucrose gradient ultracentrifugation, and chromatography on sephadex G-200.

On sucrose gradient ultracentrifugation ME-sensitive antibody activity was always found in the rapidly sedimenting fractions (19S) and ME-resistant antibody activity in the more slowly sedimenting fractions (7S), with little overlapping. Chromatography on sephadex G-200 further confirmed that the ME-sensitive antibody activity belonged to the γM class and the ME-resistant antibody activity to the γG class of immune globulins. The antibody activity of the fractions between 19S and 7S globulin were affected by ME treatment only to a small extent. It is thus likely that part of the ME-sensitive antibody activity is not exclusively due to γM-antibody and part of the ME-resistant antibody activity not exclusively due to γG-antibody, since 4 major classes of antibodies have recently been characterized in mice (6). In this discussion we will use the term γM-globulin (19S) for ME-sensitive antibody activity and the term γG-globulin (7S) for ME-resistant antibody activity, having in mind that the other classes of antibodies are included in these 2 fractions.

At a dose insufficient to completely inhibit antibody formation, 6-MP given during the primary response, selectively decreased the production of 7S antibodies and allowed the continued formation of 19S antibodies. This effect was seen in both mice and rabbits. Of particular interest is the observation that in

the 6-MP–treated animals in which the formation of 7S antibodies was markedly depressed, the synthesis of 19S antibodies continued long after this class of immunoglobulin had disappeared from the sera of control animals. These observations are in favor of a negative feedback effect of 7S antibodies on 19S antibody production. Such a mechanism has been proposed and was supported by the finding of Sahiar and Schwartz that specific 7S antibodies administered to rabbits suppressed the formation of the corresponding 19S antibodies (1).

It is also evident from these experiments that 6-MP given during the primary response has effects which extend into the secondary response. Mice which had 19S antibody in their sera as the result of 6-MP administration given with the first injection of antigen preferentially formed 19S antibody on restimulation. In control mice the secondary response is characterized by 7S antibody synthesis. It seems difficult to implicate a direct metabolic effect of the drug which has been discontinued for 10 days. Rather, this result indicates that the cell population involved in the production of 19S antibodies responds directly to the antigenic stimulus by an augmented production of γM-antibodies. This interpretation is supported by the work of Uhr who demonstrated that 19S antibody formation could be markedly increased if a second antigen injection was given 8 days after the first, at a time when circulating antibody is almost exclusively of the 19S variety (7).

The type of antibody formed in the secondary response was dependent on the dose of antigen. Control mice injected with a small dose of antigen showed a classical anamnestic response, with a rapid increase of 7S antibody. On the other hand, control mice injected with a large dose of antigen formed in addition to 7S antibodies, a second wave of 19S antibodies. This observation confirms previous experiments which demonstrated that under certain experimental conditions the synthesis of 19S antibody could be a constant feature of the anamnestic response (8).

Six-mercaptopurine given during the secondary response had no effect in mice immunized with a large dose of antigen. Ordinarily, this dose of antigen results in the production of 19S antibodies in the anamnestic response, since it was found that 6-MP has little effect on 19S antibody production. Its lack of effect under these circumstances is understandable.

In contrast to these results, 6-MP had a marked effect on mice immunized with the small dose of antigen. These animals produced a preferentially 19S immune response, while 7S antibody production did not rise as in the control animals. This suppression of 7S antibody in 6-MP–treated animals may be due to an inhibition of the rapid proliferation of plasma cells known to occur in the typical 7S anamnestic response. The reappearance of 19S antibody in this situation is most interesting. It appears possible that the feedback mechanism discussed above does not permanently suppress the formation of 19S antibody, but that once the amount of 7S antibody decreases sufficiently, cells which were

formerly producing 19S antibody are again able to produce 19S antibody following antigenic stimulation.

The secondary response of rabbits which have been treated with 6-MP during the primary and the secondary response was similar to that of mice injected with a small dose of antigen and treated with 6-MP during the secondary response only. However, in rabbits the second course of 6-MP administration resulted in the complete suppression of 7S antibody production which took place in the primary response. These animals produced exclusively 19S antibodies again. Thus it appears that 6-MP treatment is another experimental condition allowing the formation of 19S antibody in the secondary response.

19S and 7S antibody could be formed either by 2 different cell populations or by a single cell population. If γM- and γG-antibodies are produced by 2 different populations of cells, the preferential action of 6-MP on 7S antibody formation could be due either to a particular susceptibility of these cells to 6-MP or to their more rapid rate of replication, which would make them more susceptible to inhibition by 6-MP, an agent known to act preferentially on young and fast replicating cells. If the same cell population is producing first 19S and later 7S antibodies, the preferential action of 6-MP on the formation of the latter may also be explained by the difference in the rate of cell replication necessary for production of the early and late immune response. Thus recruitment of a large cell population dividing at a slow rate would be responsible for the early 19S production. A few cells would then enter in an active phase of rapid proliferation giving rise to 7S antibody producing cells. Six-mercaptopurine would inhibit this second proliferative phase of the immune response.

SUMMARY

In the primary response of mice and rabbits immunized with foreign red cells, 6-MP administration prolonged the formation of 19S antibody. 7S antibody formation was delayed and reduced in these animals.

Animals treated with 6-MP during primary response exhibited a preferential 19S response when challenged in the anamnestic response.

Animals immunized with small doses of antigen and treated with 6-MP only during the secondary response, reversed the usual antibody pattern and responded with preferential 19S antibody formation.

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