STUDIES ON ANTIBODY PRODUCTION

XII. Inhibition of Priming by Drugs*,‡

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Investigations of the immune response have included many experiments which explored the effect of various physical and chemical agents. X-Ray, nitrogen mustards, folic acid, purine, and amino acid analogues, antibiotics, and antitumor agents have all been found to have an inhibitory effect on antibody formation, and all of them are more effective in suppressing the primary than the secondary response. Of these observations, the most striking was that of Schwartz, Stack, and Dameshek (1, 2), who found that 6-mercaptopurine prevented the primary response; when the antigen dose was large, immune paralysis ensued. Comprehensive reviews have been published recently (3–5).

The development of a fixed immunization procedure in mice which results in predictable antibody responses (6) led us to test a limited number of inhibitors, especially chloramphenicol. These experiments were simultaneous with those reported from this laboratory recently on the inhibitory effect of chloramphenicol on antibody synthesis in tissue culture (7).

It was found that 6-mercaptopurine, chloramphenicol, triethylenethiophosphoramide (thio-TEPA) 8-azaguanine, and versenate all suppressed the primary response either completely or partially. But even in larger doses they had little or no suppressive effect on the secondary antibody response. In addition, each of these compounds had a partially or completely inhibitory effect on "priming" or the setting of the stage for a subsequent secondary response.

Materials and Methods

Animals.—Harvard strain adult white male mice, weighing between 25 and 30 gm, were kept in groups of 10 and fed Purina lab chow and water freely.

Antigen.—Concentrated purified diphtheria toxoid (Lot PT 105) was kindly supplied by Mr. Leo Levine of the Division of Biologic Laboratories of the Commonwealth of Massachusetts. Dilution was made with sterile saline to give a final concentration of 20 Lf per 0.4 ml. All injections were of 0.4 ml given subcutaneously along the back.

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Antisera.—Sera from about 0.5 to 0.75 ml of blood obtained by periodic bleeding of the tail artery were kept at −20°C until use. Titers were measured by hemagglutination of tanned erythrocytes sensitized with diphtheria toxoid (8). Titers are expressed as the logarithm to the base 2 of the reciprocal of ten times the highest serum dilution containing detectable antibody. Since serum dilutions of less than 1/20 were not measured for hemagglutinating antibody, non-responding mice are defined as those which failed to produce antibody detectable in serum dilutions of 1/20 or greater.

Drugs.—Triethylenethiophosphoramide (thio-TEPA), 4-amino-N^{10}-methylpteroylglutamic acid (amethopterin), and 8-azaguanine were kindly supplied by Dr. J. M. Ruegsegger, Lederle Laboratories, Pearl River, New York; 6-mercaptopurine by Dr. George H. Hitchings, Burroughs Wellcome and Company, Inc., Tuckahoe, New York; chlorpromazine hydrochloride by Mr. A. E. Palmer, Smith, Kline & French Laboratories, Philadelphia; hydrocortisone phosphate by Dr. Charles A. Winter and actinomycin D by Dr. Elmer Alpert, Merck, Sharp & Dohme, West Point, Pennsylvania; and ethidium bromide by Dr. G. Woolfe, Boots Pure Drug Co., Ltd., Nottingham, England. Chloramphenicol sodium succinate (Parke, Davis & Company, Detroit), colchicine injection (Eli Lilly and Company, Indianapolis), 4-nitroquinoline-*N*-oxide (K & K Laboratories, Jamaica, New York), versenate, calcium disodium injection (Riker Laboratories, Inc., Northridge, California) and 5-bromodeoxyuridine (California Corporation for Biochemical Research, Inc., Los Angeles) were purchased.

All drug injections were of 0.5 ml, given intraperitoneally. Solutions were freshly prepared every other day and kept in the refrigerator. Chloramphenicol, colchicine, 4-nitroquinoline-*N*-oxide, thio-TEPA, and versenate were diluted with sterile water. The water suspension of 4-nitroquinoline-*N*-oxide was filtered to remove large undissolved particles. Thio-TEPA was dissolved slowly in several drops of water before diluting. Actinomycin D, 5-bromodeoxyuridine, chlorpromazine, ethidium bromide, and hydrocortisone were diluted with sterile saline. Ten mg amethopterin was dissolved in 1 ml 0.1 N sodium carbonate and then diluted with saline. Ten mg 8-azaguanine was dissolved in 1 ml of 0.1 N sodium hydroxide and 10 mg 6-mercaptopurine in 0.06 ml of 1 N sodium hydroxide, diluted with saline, and both neutralized with hydrochloric acid.

RESULTS

Effect of Drugs upon Priming of the Antibody Response in Mice.—Fig. 1 presents the experimental plan. The experiment lasted 80 days; drug injections were given during the first 12 days only as indicated by the shaded bar. On days 0, 40, and 70, 20 Lf diphtheria toxoid was injected subcutaneously along the back, and on days 50 and 80, that is, the 10th day of the secondary and tertiary responses respectively, blood was drawn for determination of hemagglutination titer of diphtheria antibody.

The results are shown in Fig. 2. The control group of mice received saline injections during the first 12 days of the primary response. There were 31 mice in the group, of which only 2 failed to respond with a typical secondary response. The average group antibody titer was 11 (range 8 to 15). On the 10th day of the tertiary response, the average group titer was 14 (range 11 to 16).

Failure to produce detectable antibody on the 10th day of the secondary response was seen in at least 90 per cent of the mice in the thio-TEPA, chloramphenicol, and 6-mercaptopurine groups, and 55 per cent in the 8-azaguanine, versenate, and chlorpromazine groups. In each drug group inhibition of priming

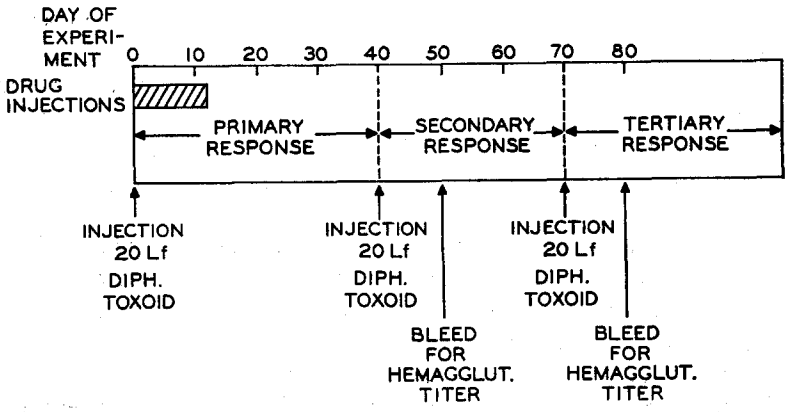

FIG. 1. Effect of various drugs upon priming of the antibody response. Experimental plan. Ten mice per group. Drug injections intraperitoneally every 8 hours; diphtheria toxoid injections subcutaneously.

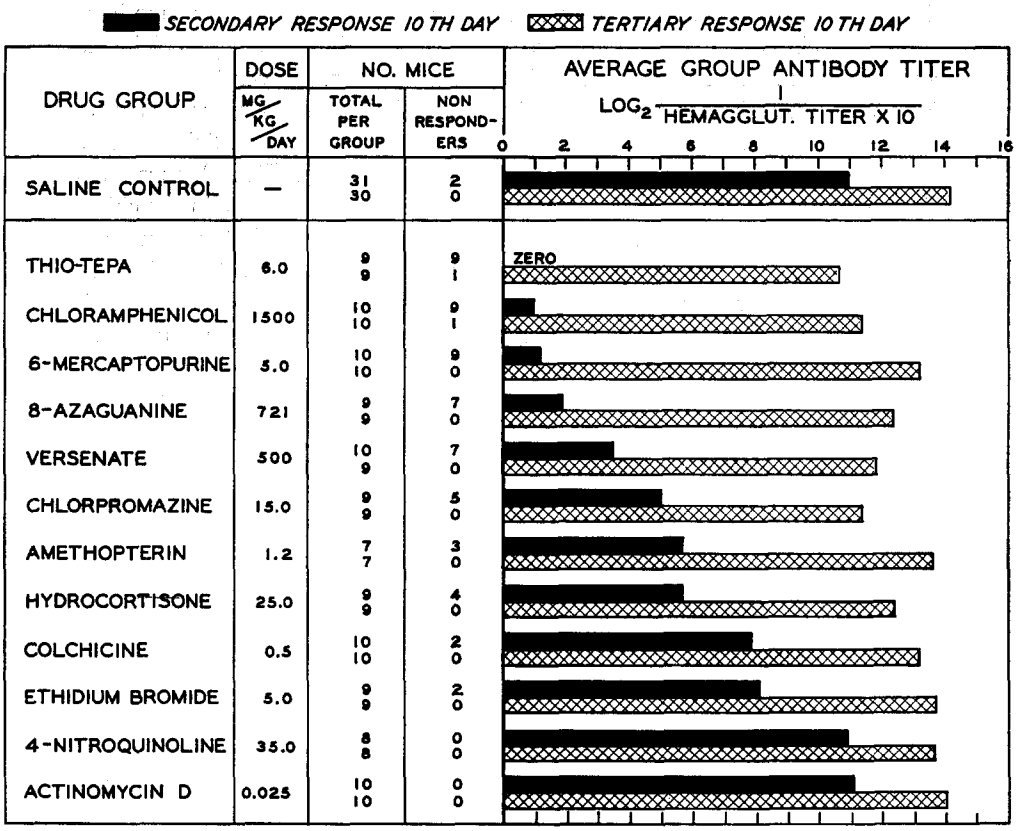

FIG. 2. Inhibition of priming of the antibody response by various drugs.

in a mouse was an "all or none" phenomenon. That is, if a mouse responded to the second antigen injection, it did so with a titer of full, predicted value. Borderline inhibition was seen in the amethopterin and hydrocortisone groups, while no inhibition was detected in the colchicine, ethidium bromide, 4-nitroquinoline-*N*-oxide, and actinomycin D groups. The average group antibody titers seen in Fig. 2 reflect, in part, the number of non-responding mice in each group.

Following the third antigen injection nearly all mice responded with detectable antibody. In general, those drug groups which had shown inhibition of priming responded with titers similar to the secondary response of the control group, whereas those groups which had not shown inhibition responded with titers similar to the tertiary response of the control group.

Effect of Drugs upon the Secondary Antibody Response in Mice.—In a preliminary report, it was suggested that drug dosages known to be effective in depressing the primary antibody response were less effective in altering the secondary response (9). In extension of these findings, the effect of altering dosage and time of administration of two possibly inhibitory drugs, amethopterin and chloramphenicol, and two possibly stimulatory drugs, colchicine and ethidium bromide, was studied. When the drugs were given for periods of 5 days or less at higher dosages, no significant alteration of the secondary response occurred, as is shown in Fig. 3. When drugs were given for 5 days before and for at least 5 days after the second antigen injection (Fig. 4), there was slight suppression of peak secondary titers of the responding mice in the chloramphenicol and amethopterin groups. There was an increase in the number of non-responders in the group given amethopterin for 10 days but when an attempt was made to give this for 15 days, the mice died on the 13th to 15th days. At lower doses both chloramphenicol and amethopterin could be given for 20 days without altering the height of the secondary response.

Fig. 5 lists the three most effective inhibitors of priming, and their effect upon the secondary response when given every 6 hours during the first 10 days of the secondary response. Doses exceeding those effective in inhibiting priming failed to alter the peak antibody titers of the secondary response. The mice receiving 16 mg per kg of 6-mercaptopurine died between the 10th and 12th day of the secondary response, presumably of drug toxicity.

Likewise mice receiving chlorpromazine (20 mg/kg/day), hydrocortisone (50 mg/kg/day), or colchicine (1.2 mg/kg/day), every 6 hours for 10 days of the secondary response failed to show inhibition of antibody formation when compared to control groups. Mice given 5-bromodeoxyuridine (1000 mg/kg/day) developed marked signs of toxicity and so the drug was discontinued on the 5th day of the secondary response. The average titer of the 5 responding mice on the 7th day was 7.8 and the 2 mice who were non-responders died on the 10th day. Therefore, there may have been slight suppression of the antibody response.

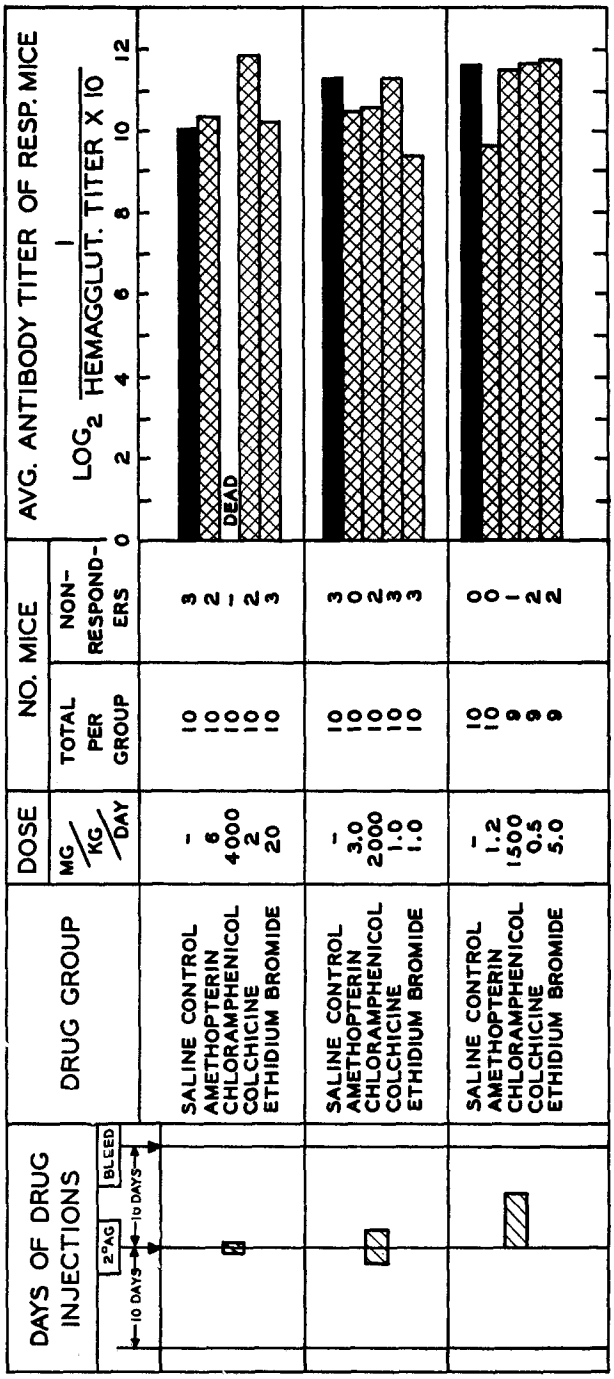

FIG. 3. Effect of drugs on the secondary antibody response. The interval between the first and second antigen injection was 40 days. Drug injections were given intraperitoneally every 8 hours. 2° AG, second dose of antigen.

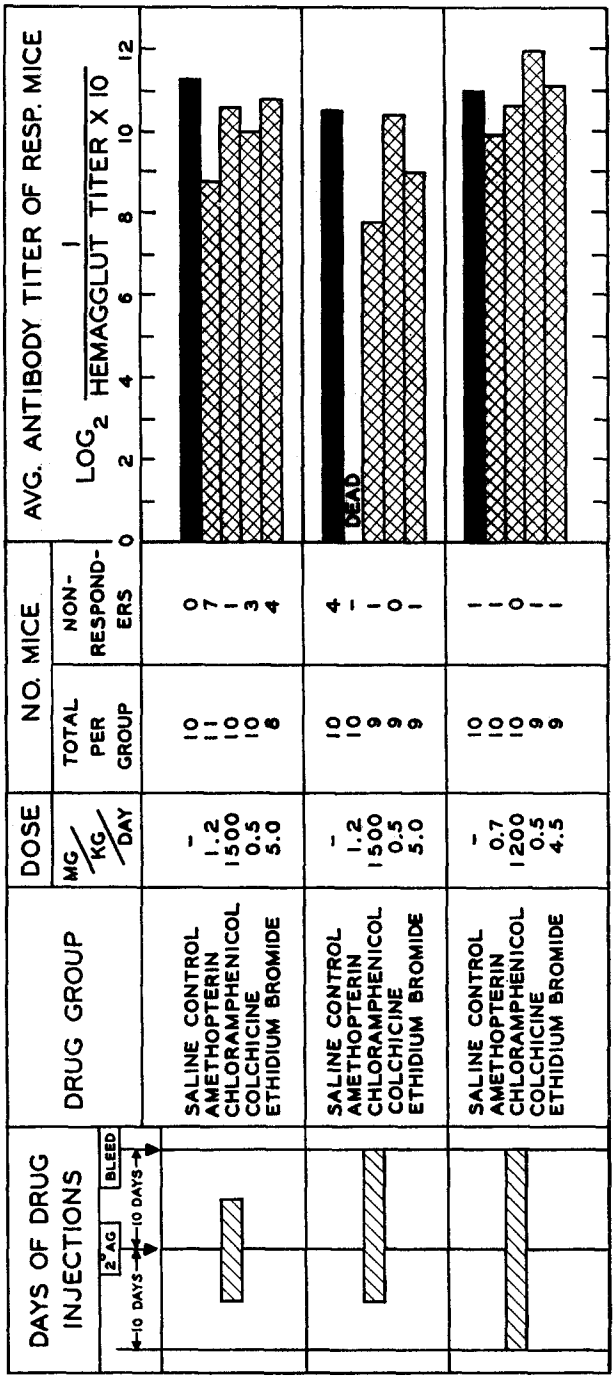

FIG. 4. Effect of drugs on the secondary antibody response. The interval between the first and second antigen injection was 40 days. Drug injections were given intraperitoneally every 8 hours. 2° AG, second dose of antigen.

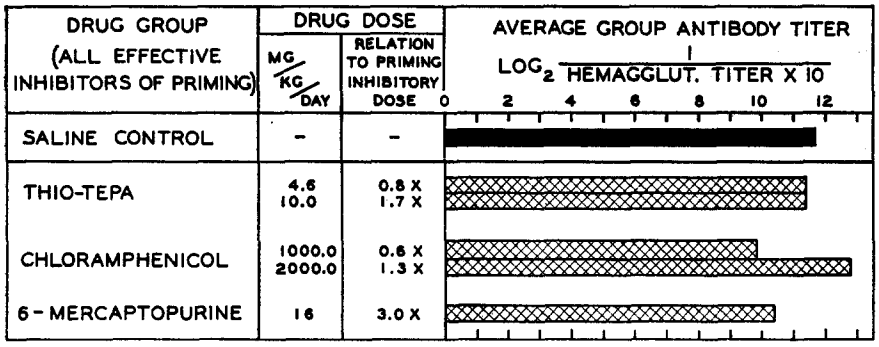

FIG. 5. Effect of drugs on the secondary antibody response. Ten mice per group. The interval between the first and second antigen injection was 40 days. Drug injections were given intraperitoneally every 6 hours throughout the secondary response.

DISCUSSION

The mouse system used in this study has been described elsewhere (6). It employs a uniform antigen dosage and spaces antigen injections at an interval of 40 days; the average antibody titer of the group at the peak of the secondary response is predictable. Therefore, we could examine the effects of drugs not only by comparison with simultaneous control groups receiving saline, but also with the standard response.

The drugs utilized in the experiments were chosen for their ability to do one or more of the following: to alter the antibody response in mice or rabbits, to depress mouse tumor formation, or to inhibit protein synthesis *in vivo* or *in vitro*. The drug dosages given were, in general, close to the maximum that could be tolerated without causing immediate or delayed death. It was observed that mice did not tolerate the same drug dosages given in portions every 6 or 8 hours that other authors had given once daily. During injection periods, nearly all the mice in the drug groups were unhealthy in appearance as evidenced by lethargy, discoloration or loss of hair, diarrhea, and weight loss. During a 10 day drug injection period the average loss of body weight was 15 per cent in the hydrocortisone and 8-azaguanine groups, between 8 and 12 per cent in the chloramphenicol, 4-nitroquinoline-*N*-oxide, ethidium bromide, and thio-TEPA groups, less than 8 per cent in the remaining drug groups, and 1 per cent in the control groups.

The effect of drugs given during the first 12 days of the primary response was determined by comparing the height of the secondary response with the standard response. If, for instance, no antibody was demonstrable during the secondary response, we concluded that the mice had been made indifferent to the initial injection of antigen by the presence of a drug. Whether this in-

difference was temporary or permanent was determined by giving a third injection of antigen and measuring the tertiary response.

In such experiments, there were varying degrees of inhibition of priming, ranging from complete inhibition in the thio-TEPA group to no significant inhibition in the colchicine, ethidium bromide, 4-nitroquinoline-*N*-oxide, and actinomycin D groups. The thio-TEPA mice, therefore, had been made indifferent to the presence of diphtheria toxoid during the period of the drug injections. The response of the thio-TEPA group to the third antigen injection was similar to the response of the control group to its second antigen injection. Therefore, the failure of priming was temporary and the drug injections did not alter the ability of the mice to respond to later antigen injections in predicted fashion.

In addition to thio-TEPA, the drugs most strongly inhibiting priming of the antibody response were chloramphenicol, 6-mercaptopurine, 8-azaguanine, and versenate. Partial inhibition was seen in the chlorpromazine, amethopterin, and hydrocortisone drug groups. These results are in general agreement with those of other authors, except that chloramphenicol has not been shown previously to inhibit so strongly antibody formation *in vivo*.

The drugs found to be effective in inhibiting priming were also administered during a secondary response; there was no significant alteration of the response. When chloramphenicol and amethopterin were administered for at least 5 days prior to the second antigen injection there was, however, a suggestion of suppression of peak secondary titers in responding mice. It appears, therefore, that agents effective in suppressing priming are less effective in altering the secondary response.

Discrepancy between the present experiments and other reports indicating suppression of the secondary response by nitrogen mustard (10), 6-mercaptopurine (11), and hydrocortisone (12) may be explained by a difference in animal species, antigenic stimulus, and by the duration of drug administration and dosage. For example, LaPlante *et al.* were able to suppress the secondary response in rabbits with 6-mercaptopurine when they gave it in doses of 12 to 15 mg/kg (11). It is interesting that thio-TEPA, a powerful inhibitor of cell division, should have so little effect upon the secondary response in spite of the fact that mitoses play an integral part in it (13).

Antibody formation during the secondary response in mice was *less* sensitive to alteration by various pharmacological agents than other aspects of protein metabolism as manifested by generalized toxicity. For instance, the two drugs most effective in producing weight loss, hydrocortisone and 8-azaguanine, failed to alter the secondary response. This lack of correlation has also been noted before (14).

That the effect of drugs on the antibody response in mice depends in part upon whether the animal has had previous contact with the antigen seems to

imply once again a fundamental difference between the primary and secondary antibody responses.

SUMMARY

The effect of drugs upon the primary and the secondary antibody response to diphtheria toxoid in mice was studied using an experimental system previously described.

Triethylenethiophosphoramide (thio-TEPA), chloramphenicol, 6-mercaptopurine, 8-azaguanine, and versenate were found to inhibit, partially or completely,"priming" for the secondary response.

Thio-TEPA, chloramphenicol, and 6-mercaptopurine, in doses exceeding those effective in inhibiting priming, did not cause alteration of the secondary response when given only during the secondary response. However, when chloramphenicol and amethopterin were given for 5 days prior to and at least 5 days after the second antigen injection, slight suppression of peak secondary titers occurred.

Therefore, drug dosages effective in suppressing priming had less effect on the secondary response. It thus appears that there is a real difference between "priming" and the induction of antibody synthesis.

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