Inhibition of Hemagglutinin Formation by Thioguanine: Dose - Time Relationships.* (27544)

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During experiments dealing with inhibition of hemagglutinin formation by thioguanine, it was observed that pretreatment of mice with drug had no effect on the primary response (1). Heretofore, we had assumed that TG acted by destroying cells involved in antibody synthesis because of its marked cytopathogenicity for myeloid and erythroid elements despite the fact that Philips *et al.*(2) had found that plasma cells were resistant. From our experiments on pretreatment, it was concluded that the concept of cell destruction was not tenable and that a metabolic block was the more likely mode of action of TG. Data bearing on this concept are reported herein. The methodology has been described(3,4).

In the first experiment, groups of 10 mice were given 0.2 ml of washed and packed OD cells intraperitoneally, followed by a single intraperitoneal dose of 20 mg/kg TG in 0.25% methocel at the times shown in Fig. 1. Blood samples were obtained from the orbital sinus and individual serums were titered at 6, 10 and 15 days following antigenic stimulus. The mean hemagglutination titer for each group was determined for the particular test day. In Fig. 1 and in all of the others, a value of 1 log to the base 2 indicates that hemagglutinins were not detected in the initial serum dilution of 1:8. The mean titers of the untreated animals are shown at point K on the y axis and are to be compared with the test animals on the right. The curves show that significant inhibition was obtained when TG was injected 24, 36, 48, 60 and 72 hours after primary immunization but not at the 2- and 12-hour intervals. The control titer reached a peak at 6 days and fell slightly over the next 2 test periods. Inhibition by TG was greatest on the 6th day with some recovery occurring on the 10th and 15th day. Since previous studies(1) had indicated that TG given 96 hours after primary immunization was ineffective, extension of the time was not attempted.

We next determined the effect of varying doses of TG on the course of inhibition. For this purpose, groups of 6 mice received 0.2 ml of washed and packed OD$^+$ cells intraperitoneally. At intervals thereafter, shown in Fig. 2, they were given a single dose of either 10, 15 or 20 mg/kg of TG in 0.25% methocel intraperitoneally. Individual blood samples were obtained from the orbital sinus and titered 4, 6 and 10 days following antigenic stimulus. Although some inhibition of antibody formation occurred when TG was injected at 6 and 15 hours after primary immunization, particularly with the 20 mg/kg dose, the major drug effect was observed at the 24- and 36-hour intervals.

Because of the relatively large doses of drug used, we could not eliminate the possibility that the TG given at the 6- and 15-hour intervals may have persisted in the tissues until the critical period when the antibody forming system was most susceptible to inhibition. For this reason, the dose of TG was reduced in the next experiment. Groups of 5 mice were immunized with 0.2 ml of washed and packed OD$^+$ cells given intraperitoneally. At intervals, 2 groups received a single intraperitoneal dose of 2.5 and 5 mg/kg of TG in 0.25% methocel respectively. Blood samples from the orbital sinus were obtained 7 and 14 days following primary immunization. The mean hemagglutination titers are shown in Fig. 3. With minimal amounts of drug, the inhibition was less marked than had been noted previously. A distinct effect, however, was obtained when the TG was given 18 hours after antigenic stimulation with a dose as small as 2.5 mg/kg. Partial recovery occurred when the drug was given at 24 and 36 hours. Administration of TG at 0 and 6 hours after antigenic stimulation did not appreciably influence the mean titer; there was sug-

* Supported by a grant from Nat. Inst. of Allergy and Infect. Dis., Nat. Inst. Health, U. S. P. H. S.

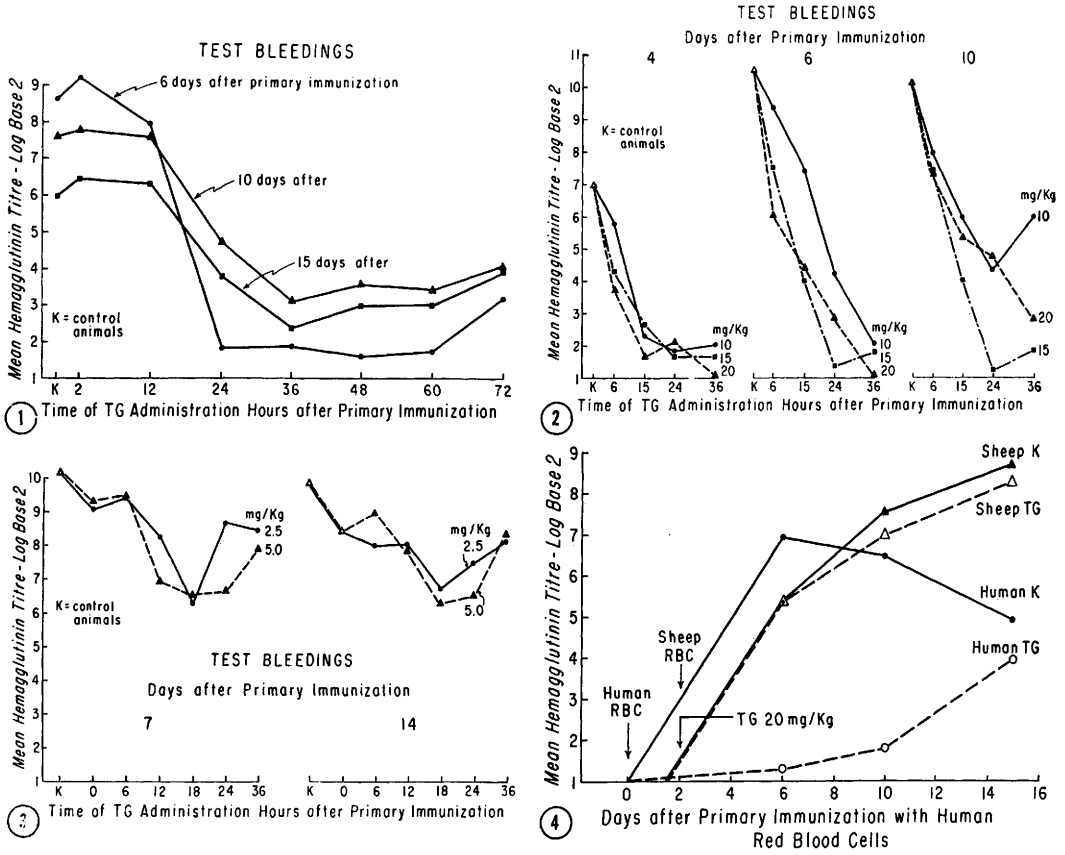

FIG. 1. Inhibition of hemagglutinin synthesis by one dose of 20 mg/kg TG given at varying times after primary immunization.

FIG. 2. Inhibition of hemagglutinin synthesis by one dose of 10, 15 or 20 mg/kg of TG given at varying times after primary immunization.

FIG. 3. Inhibition of hemagglutinin synthesis by one dose of 2.5 or 5 mg/kg of TG given at varying times after primary immunization.

FIG. 4. Inhibition of anti-human but not anti-sheep hemagglutinin synthesis by a single dose of 20 mg/kg of TG.

gestive but not conclusive depression when the drug was given after 12 hours.

Because of the delicacy of the system under study, it was decided to determine if the same mice were capable of demonstrating inhibition of one antibody while synthesizing another. For this purpose, groups of 10 mice were given 0.2 ml of washed and packed group OD^+ cells intraperitoneally. Thirty-six hours later, half of them received 20 mg/kg of TG in 0.25% methocel intraperitoneally. All mice were then given 0.2 ml of washed and packed sheep red cells intraperitoneally. Individual mice were bled at 6, 10 and 15 days after immunization with OD^+ cells and titrated for human and for sheep hemagglutinins, the corresponding intervals for sheep being 4½, 8½ and 13½ days as shown in Fig. 4. It is clearly evident that anti-human hemagglutinin formation was blocked by TG but that anti-sheep hemagglutinins were not, at all, affected.

Discussion. One of the characteristics of antibody inhibition by cortisone, X-irradiation, and nitrogen mustard is the fact that all 3 agents are effective if given prior to antigenic stimulation. For cortisone, this is mandatory(5). In the rabbit, the time period of 24 hours following antigen administration is most radio sensitive(6). Rats, however, will show complete suppression of antibody synthesis if they are treated from 1 to 6 days

before antigenic stimulation(7). Mice are also sensitive to pre-irradiation(8). The nitrogen mustards inhibit the induction period when given 3-4 days prior to antigen but can also influence peak titers on injection 48 hours after antigen(9). In the case of thioguanine, pretreatment for as long as 6 days has no effect, whatsoever, on antibody synthesis(1). It would appear, therefore, that the mechanisms of action of the purine analogues at an immunological level is distinctly different from cortisone, X-ray and nitrogen mustards.

From cell-transfer studies, Sterzl concluded (10) that 6-MP interferes with the inductive phase of antibody synthesis during the first 48-72 hours after antigenic stimulation. The data presented here indicate that the antibody forming process continues normally in the presence of TG during the first 12 hours. During this period, the antigen circulates and undergoes phagocytosis by cells which either initiate the antibody response directly, or transfer the information to other cells. None of these processes appear to be interfered with by 6-MP(11,12) and, presumably, not by TG. Division and multiplication of antibody forming cells is generally believed to be the next step in the process, and it seems unlikely that either 6-MP or TG interfere at this point. Thus Sterzl has shown that highly antimitotic drugs, such as colchicine and actinomycin C, in maximal doses do not prevent antibody synthesis in the system used by him whereas 6-MP does(13). Finally, Schwartz and co-workers(12) have pointed out that 6-MP does not interfere with protein synthesis *per se* in hyperimmunized animals. With the dose of antigen employed in these experiments, circulating antibody begins to appear within 72 hours following primary immunization. The data which we have presented indicate that TG induces a metabolic block which is selective for the last two-thirds of the induction phase. Whether this involves a failure of transfer of information, interference with replication at the template, temporary inhibition of synthesis, or distortion of the antibody molecule remains to be determined.

The experiment involving simultaneous inhibition of one type of antibody by TG and normal synthesis of another, as shown in Fig. 4, may serve as a useful model for the study of antibody formation at a cellular level. One could postulate that 2 separate "clones" of cells are involved. However, additional experiments will be necessary to demonstrate that all anti-human receptors are completely blocked. It is also possible, but unlikely, that the same cells may have been responsible for the formation of both varieties of hemagglutinins. It would appear, however, that TG does leave at least a portion of the antibody forming apparatus intact and capable of reacting to a primary stimulus which cannot be differentiated from the normal. The mice must, therefore, be considered immunologically competent to produce anti-sheep red cell agglutinins at a time when their anti-human red cell agglutinin capacity is markedly reduced. Studies with this model are being continued.

Summary. Inhibition of hemagglutinin formation in mice was demonstrated with single doses of 2.5, 5, 10, 15 and 20 mg/kg of TG. If the drug was administered 18 or more hours after primary immunization, maximal block of antibody synthesis occurred. The minimal effective dose was 2.5 mg/kg injected 18 hours after antigenic stimulation. The same amount of drug given either before or after was ineffective. The timing was so specific that it permitted the design of a model system in which simultaneous inhibition of anti-human hemagglutinins was obtained without significant interference with the formation of anti-sheep hemagglutinins. The significance of the data are discussed.

The technical assistance of Mr. John Niedra is gratefully acknowledged.

1. Frisch, A. W., Davies, G. H., Milstein, V., *J. Immunol.*, 1962, in press.

2. Philips, F. S., Sternberg, S. S., Hamilton, L., Clarke, D. A., *Ann. N. Y. Acad. Sci.*, 1954, v60, 283.

3. Frisch, A. W., Davies, G. H., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 281.

4. ———, *J. Immunol.*, 1962, v88, 269.

5. Berglund, K., *Acta Path. Microbiol. Scand.*, 1956, v38, 311.

6. Taliaferro, W. H., *Ann. N. Y. Acad. Sci.*, 1957, v69, 745

7. Fitch, F. W., Wissler, R. W., LaVia, M., Barker, P., *J. Immunol.*, 1956, v76, 151.

8. Gengozian, N., Makinodian, T., *ibid.*, 1958, v80, 189.

9. Green, D. M., *Brit. J. Exp. Path.*, 1958, v39, 192.

10. Sterzl, J., *Nature*, 1960, v185, 257.

11. Schwartz, R., Eisner, A., Dameshek, W., *Clin. Res.*, 1959, v7 39.

12. ———, *J. Clin. Invest.*, 1959, v38, 1394.

13. Sterzl, J., *Nature*, 1961, v189, 1022.

Received April 26, 1962. P.S.E.B.M., 1962, v110.

Specific Immunologic Unresponsiveness to Delayed Hypersensitivity Elicited in Adult Mice.* (27545)

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During experimentation on factors affecting induction of delayed hypersensitivity to protein antigens in mice, we have encountered a form of specific immunologic unresponsiveness affecting this delayed hypersensitivity, of the tuberculin type, but not immediate hypersensitivity, and which is induced in immunologically mature animals. The way in which it is elicited is unique and apparently has not hitherto been described. It is to treat animals which have received a hypersensitizing injection with a solution of antigen during a critical short period after that injection. Preliminary description of this finding is presented here because it aids in distinguishing mechanistically between immediate and delayed hypersensitivities, and because its experimental application to various immunological problems may help to explain the events which occur after vaccination and lead to development of humoral and cellular antibody allergies.

Materials and methods. CF_1 white female mice 6 to 8 weeks old maintained on water and Rockland mouse pellets were employed. The experimental antigen was twice-crystallized chicken ovalbumin (OVA) purchased from Nutritional Biochemicals Corp. Living, avirulent H37Ra strain tubercle bacilli cultured on the surface of Kirchner synthetic medium were utilized as mycobacterial adjuvant. For the hypersensitizing injection these two materials were incorporated in a water-in-oil (w/o) emulsion of a composition and by technics described elsewhere(1). Skin tests were performed by intracutaneous injection of 0.02 ml volumes of 1% OVA freshly prepared in physiologic phosphate buffer of pH 7.4(2). Skin reaction diameters were read with calipers 3 hours and 24 hours after tests were performed representing, respectively, immediate and delayed hypersensitivities(1, 3), and results are expressed in mean millimeters of reaction diameter for each group of animals. These reactions are evident as skin thickenings, edema for immediate hypersensitivity and induration for delayed hypersensitivity. In mouse skin erythema commonly is absent from such reactions(2).

Experiments and results. The mice were divided into 2 large groups. One group was given no sensitizing injection but was subdivided into several smaller groups of 10 to 12 mice each to provide nonallergic skin-test control animals. Each mouse in the second group received a single subcutaneous injection into the groin of 0.1 ml of w/o emulsion containing 0.25 mg of OVA (dry weight) together with 0.25 mg of bacilli (moist weight). Then this group was divided into 5 subgroups, also of 10 to 12 mice each, so that reactions to primary skin tests could be measured at 4, 8, 12, 18, and 22 days after vaccination. Each group after it had received its first test was skin-tested again at every succeeding test period. For example, mice in the group tested 4 days after vaccination also were tested at 8, 12, 18, and 22 days. Succeeding skin tests

* Supported by research grants from Nat. Inst. Health.