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Depression of the Primary Immune Response by dl-Penicillamine.* (29905)

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Penicillamine (dimethyl cysteine), one of the mercaptan compounds, has been introduced as a therapeutic agent for a number of clinical entities. Its property as a chelating substance has been used to induce cupruresis in Wilson's disease as shown by Walshe (1). The SH-radical of penicillamine is active in the chelation of copper and other heavy metals and appears to be effective also in the depolymerization of macroglobulins *in vitro*(2,3) and *in vivo*(2,4,5). The effectiveness of penicillamine in reducing the clinical syndromes associated with macroglobulinemia of Waldenström's disease(4), rheumatoid arthritis(6), and autoimmune hemolytic anemia(3,7) warrants basic studies of its effect on an experimentally induced antibody-synthesizing mechanism to determine its mode of action. Tobin and Altman(8) investigated the influence of dl-penicillamine on the immune response of white New Zealand rabbits to human serum albumin and found an acceleration of the antibody production with corresponding earlier clearance of the antigen. The present study was undertaken to test the effect of a prolonged exposure to dl-penicillamine on the antibody response in mice injected with *S. typhosa*, an antigen that stimulates, in most species, a macromolecular antibody in the early phases of a primary immune response.

Materials and methods. Animals. Male $C3BF_1$ [$(C3H \times C57B1)F_1$] mice, approximately 13 weeks old, were used in all experiments. They were fed food and water *ad libitum*.

Penicillamine. The dl- form of penicillamine‡ was used. The solution was made up immediately before use in phosphate buffered saline and the pH was adjusted to 7.2. Preliminary toxicity studies showed that intraperitoneal injections of 2.5 mg daily or 5.5 mg every other day into 25-g mice could be given safely. Single intraperitoneal injections of 25, 20, 15, and 10 mg were found to be lethal in 12 hours. For the present study, the dose for each injection was 5.5 mg in 0.5 ml given subcutaneously (s.c.) or intraperitoneally (i.p.), the injection regimen being either daily (q.d.) or every other day (q.o.d). The penicillamine treatment relative to the antigen injection for various groups is shown in Table I. Only in Group B, which received the penicillamine i.p. for 8 consecutive days, was mortality high. Here 12 of 20 animals died in 3 days after penicillamine treatment

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TABLE I. Penicillamine Treatment Schedule Relative to Antigen Injection.

Group	No. of animals	Day −21 to day 0	Day 0	Day 0 to day +23
A	10	No treatment	Ag	No treatment
B	10	" "	"	5.5 mg q.d. (×8), i.p.
C	20	5.5 mg q.o.d., i.p.	"	No treatment
D	20	*Idem*	"	5.5 mg q.o.d., i.p.
E	10	5.5 mg q.d., s.c.	"	5.5 mg q.d., s.c.

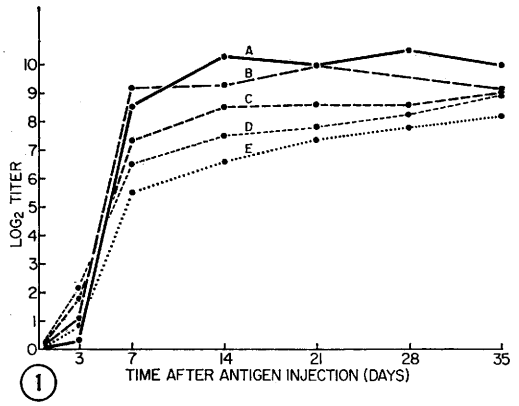
was begun. Additional numbers were therefore injected to provide at least 10 animals that could be studied at the desired time intervals after antigen injection.

Immunization. Immunization was done with the typhoid H antigen *(S. typhosa)* made by Markham Laboratories (Chicago, Ill.) as a diagnostic antigen for tube test titrations. This antigen preparation was used because preliminary studies showed that it induced an excellent primary antibody response in normal mice, greater than could be obtained with the commercially available typhoid vaccine. Ten to 20 animals were used in each experimental group designated in Table I. The antigen was given in a single total dose of 0.5 ml, 0.1 ml into each inguinal area s.c., and 0.3 ml i.p. Blood samples were taken from the sinus cavernosus of each animal on days 3, 7, 14, 21, 28, and 35 after antigen injection. Serum samples were frozen at −20°C until titrated.

Antisera titrations. Serum samples from each animal were titrated individually. Twofold serial dilutions of 0.1 ml serum were made in saline, followed by addition of 0.1 ml of the typhoid H tube-test antigen. The reaction mixture was incubated at 56°C for 2 hours and at 4°C for approximately 18 hours. Titers were expressed as the $\log_2$ of the reciprocal of the highest dilution giving visible agglutination. Statistical evaluation of the data was made by analyses of variance and the Student t test.

Treatment with the sulfhydryl compound, 2-mercaptoethanol (2-ME), of antibody macroglobulins (sedimentation coefficients of 15 or more) has been shown to result in dissociation of the protein into smaller subunits with loss of antibody activity(9). Two-ME treatment of the smaller 7S antibody gamma globulins, on the other hand, does not result in any loss of antibody activity. The sera obtained in this study were therefore treated with 2-ME to determine the sequential formation of both the heavy and light antibody after stimulation with typhoid antigen. For convenience these will be referred to as 19S and 7S antibodies, respectively, although sedimentation coefficients were not determined. Treatment with 2-ME was as follows: the serum was diluted 1:1 with phosphate buffered saline (pH 7.2) and to it was added an equal volume of 0.2 M 2-ME, to give a final serum dilution of 1:4 and 2-ME concentration of 0.1 M. The mixture was incubated at 37°C for 1 hour and room temperature overnight. The samples were then titrated in the usual manner, without dialysis for removal of the 2-ME since preliminary studies had shown essentially no difference in titer. Treatment with dl-penicillamine had the same effect as 2-ME in inactivating the macroglobulin antibody *in vitro* as noted by others(2,3).

Results. Fig. 1 shows the typhoid H agglutinin titers for the normal and experimental groups. Fig. 2 shows the agglutinin titers for the same groups after treatment of the sera with 2-ME. Considering first the total antibody formed (*i.e.*, non-2-ME treated sera, Fig. 1) from the 7th through the 35th day after antigen injection, a response significantly lower ($p<0.001$) than the normal group A was obtained in 3 groups, C, D, and E. The depression was greatest in Group E, which had received daily subcutaneous injections of penicillamine before and after antigen injection (Table I). Intraperitoneal injections of penicillamine q.o.d. before antigen also reduced the immune response (Group C), the reduction being slightly less ($p<0.05$) than that obtained in animals (Group D) with similar treatment continued after antigen injection. Among the 4 experimental groups, only B showed a response comparable to the normal, and this group received penicillamine treatment only *after* antigen injection (Table I). Although the responses in all



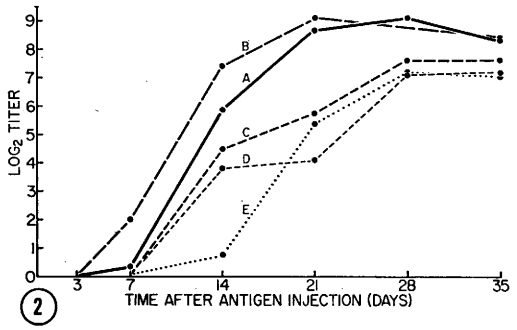


FIG. 1. Agglutinin response to typhoid H antigen in normal mice and mice treated with dl-penicillamine. (See Table I for legend.)

FIG. 2. Agglutinin titers following treatment of the immune sera with 2-mercaptoethanol. (See Table I for legend.)

groups were minimal on day 3 after antigen, there is some suggestion that penicillamine pretreatment may have induced an early immune response, noting particularly Groups C and D relative to the normal control Group A.

The production of 7S antibodies was both delayed and reduced in Groups C, D, and E ($p<0.001$) as noted by the titrations following 2-ME treatment of the sera (Fig. 2). This was most pronounced in Group E where significant amounts of the 2-ME resistant antibody was not produced until the 21st day after immunization. The sharp rise in 7S-type antibody from day 14 to day 21 in Group E was attained while penicillamine was still being administered to the animals. A similar rise in the 7S antibody was noted in Group D between the 21st and 28th day after antigen injection. To see if this could be prevented by continued penicillamine treatment, additional animals were treated similarly to Group D with the exception that the penicillamine injections were continued through day 35. The antibody titers (both 2-ME and non-2-ME) of this group did not differ from that seen in Group D on days 28 and 35 (data not shown). In terms of the 7S antibody production, Group B compared favorably to the normal control Group A, and indeed showed a significantly greater response on day 14 ($p<0.02$).

In an attempt to demonstrate *in vivo* intravascular degradation of circulating macromolecular antibodies by penicillamine, a group of 20 animals was injected intravenously with 10 mg of penicillamine in 0.5 ml of saline on the 6th day after antigen injection, a time when all antibody is considered to be of the 19S variety. Ten were killed 1 hour after penicillamine injection and the 6 remaining survivors, 24 hours later. One control group received 0.5 ml saline and another was not injected. As shown in Table II, 1 hour after penicillamine the serum titer was reduced significantly ($p<0.001$), although large amounts of antibody were still present in the serum. Animals bled 24 hours after penicillamine treatment showed no further reduction but rather a slight rise (but still below the normal expected for day 7), indicating that the effect of penicillamine was immediate and transient. Increasing the dose of penicillamine to 20 mg, 30 mg, and 40 mg did not decrease the serum titer beyond that seen with 10 mg at 1 hour after injection

TABLE II. Effect of Intravenously Administered dl-Penicillamine on Serum Macroglobulin Antibody.

Group*	No. of animals	Treatment	Antibody titer ($\log_2$)†
I	10	None	7.6
II	10	.5 ml saline 1 hr before sacrifice	7.6
III	10	10 mg (.5 ml) dl-penicillamine 1 hr before sacrifice	5.8
IV	6	10 mg (.5 ml) dl-penicillamine 24 hr before sacrifice	7.0

* All animals injected with *S. typhosa* H antigen 6 days before study.

† Groups I and II *vs* Group III, $p<.001$ by t test.

(data not tabulated). The high mortality attendant with the various doses of penicillamine administered intravenously prohibited studies beyond this 1-hr period.

Discussion. The clinical use of penicillamine in macroglobulin-associated immune disorders was motivated by its *in vitro* ability to depolymerize and inactivate proteins through its sulfhydryl radical. That this could be effected *in vivo* was demonstrated by Bloch *et al*(4) in the serum of a patient with Waldenström's disease, and by Jaffe(2) in the synovial fluid of rheumatoid arthritis patients. Ritzman and Levin(7) reported that dl-penicillamine treatment decreased significantly the macroglobulin antibody in a case of cold agglutinin disease, and that 6-7 weeks after penicillamine therapy was discontinued the antibody titer returned to pretreatment levels. Their tentative interpretation was that penicillamine exerted its beneficial effects by *in vivo* intravascular dissociation of the cold agglutinin. On the other hand, Jaffe(6), in 2 cases of rheumatoid arthritis, and Edwards and Gengozian(3), in a case of autoimmune hemolytic anemia, reported a continued suppression of the serum macroglobulins after cessation of oral dl-penicillamine therapy, suggesting penicillamine to be effective also at the cellular level of the immunologic process.

The studies undertaken here, in mice actively immunized against an antigen that induced formation of both 19S and 7S antibodies, have shown that dl-penicillamine 1) has a depressive effect on the immune mechanism, and 2) that this depression is mediated primarily at the cellular level rather than through any intravascular dissociation process. This latter conclusion is based primarily on data showing a) the absence of any effect on the immune response when penicillamine was administered *after* antigen injection for 8 consecutive days, a time when the macroglobulin antibodies are being formed, and b) the delay and reduction in titer of the 7S type antibody in all groups treated with penicillamine *before* antigen injection. The lower levels of this smaller antibody protein in the serum could not be due to any intravascular degradation, but rather point to a penicillamine effect on the potential antibody forming cells *per se*. The inability of penicillamine to suppress synthesis of the 7S antibody after antigen injection further emphasizes the requirement for exposure of the cells to penicillamine before antigen stimulation. The immediate and only transient effect of intravenously administered penicillamine on serum macroglobulin antibodies also argues against any significant intravascular dissociation process to account for the lowered serum titers under the conditions of the experiments reported here. The transiency of this effect is in agreement with Walshe's findings in patients with Wilson's disease showing a rapid excretion of penicillamine and a rather abrupt reduction of the cupruresis as soon as administration of the chelating penicillamine is stopped(1). Jaffe(2) also demonstrated a transient effect of penicillamine following intra-articular injections in patients with rheumatoid arthritis and synovitis. Within 24 hours after injection, the rheumatoid factor in the synovial fluid was returning to pretreatment levels. It is possible that in those mice maintained on dl-penicillamine before and after antigen injections some *in vivo* dissociation of the macroglobulins occurred because of accumulation of the drug in the system. This, however, does not appear to be a major contributory factor, for a significant depressive effect was noted if penicillamine was administered only before antigen injection.

The mechanism through which penicillamine exerts its depressive effect on the immune system is not known. Kuchinskas and du Vigneaud(10) have shown growth inhibition in rats treated with l-penicillamine that could be reversed with higher levels of pyridoxine in the diet or by injection of the vitamin. They suggested that l-penicillamine may interfere with the metabolic effects of the vitamin by combining with the coenzyme pyridoxal 5-phosphate and thus reduce its participation in certain enzyme systems. That vit. B_6 deficiency *per se* can cause a reduction in the immunologic capabilities of animals is well documented(11,12,13). It is thus possible that the antibody depressing effect of dl-penicillamine as shown here is

mediated through vit. B_6-dependent enzyme systems. This postulate, however, makes more obscure the relation, if any, between penicillamine and the macroglobulin antibody protein formed in the initial phases of a primary immune response. Thus, the rationale for the present study was based on the depolymerizing effect of penicillamine on macroglobulins *in vitro* and *in vivo* and its effectiveness in clinical cases involving macroglobulin-associated disorders. Whether there occurs an intracellular effect of penicillamine on the synthesis of macroglobulin that in turn reduces the efficiency of the antibody-forming mechanism must be determined.

Our data do not allow for a definitive statement concerning an accelerated immune response by penicillamine as reported by Tobin and Altman(8). In their study rabbits were injected intramuscularly with dl-penicillamine (75 mg/kg body weight) every other day for 6 weeks. Human serum albumin was injected intravenously on the 28th day and the serum was tested for antigen and antibody for 11 consecutive days after injection. Their results showed an earlier clearance of antigen along with an earlier formation of antibody in the penicillamine-treated animals. Whether their experiments, however, would have resulted in an equal, greater, or lesser amount of antibody formed subsequent to the 11-day period following antigen injection is not known. Thus, although there was some suggestion of increased antibody formation in our study in 2 penicillamine-treated groups on the 3rd day after antigen injection, subsequent tests showed an overall reduced response. The major variable between their experiments and ours, however, in addition to the different species of animal and type of antigen used, was the dose of penicillamine. In the present study, 5.5 mg of dl-penicillamine given at each injection was equivalent to approximately 220 mg/kg body weight, a dose 3 times as large as that used in their study.

Summary. Treatment of mice with dl-penicillamine for 3 weeks before antigen injection resulted in an overall reduction in antibody formation while treatment immediately after antigen injection for 8 consecutive days had no effect. In those groups showing depression of antibody response, the formation of both the 19S and 7S antibody was reduced, the latter type also showing a delay in appearance but subsequently reaching levels comparable to those seen in normal animals. Administration of penicillamine intravenously to animals having only 19S antibody in the serum resulted in an immediate but transient reduction of antibody titer. This observation, along with that showing failure of penicillamine to affect the formation of both 19S and 7S antibody when given after antigen injection, suggests that the depressive effect is primarily at the cellular level of the immunologic process rather than through an intravascular dissociation process.

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