Antibody Response Following Injection with *M. tuberculosis.** (32559)

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The importance of route of inoculation in producing antibodies has been well established(1-5). These studies also show that the antibody response is dependent on several variables: nature of the antigen, dose of antigen, species of animal inoculated, and test employed to detect the antibody. Similar studies employing *M. tuberculosis* as antigen have not been reported.

The literature concerning antibody response in experimental tuberculosis is difficult to evaluate because of differences in the dosage of organisms employed, the route of administration, the experimental animal utilized, and the serological tests employed(6-9). Castelnuovo and Marellini(10) have pointed out that these discordant results might be due in great part to the use of different kinds of antigen preparations, serologic techniques, and procedures for the production of antisera to *Mycobacterial* antigens.

In general, studies on the nature of the immunoglobulin response to a single injection of antigen show that 19S globulin appears first and is then followed by the appearance of 7S globulin(11,12,13). However, there are some exceptions to this general finding(14). Immunization of human beings with typhoid O antigen stimulates production of 19S antibodies(15); however Pike *et al*(16) have shown both 19S and 7S antibody in rabbits injected with typhoid O antigen.

This paper reports studies with *Mycobacterium tuberculosis* antigens, employing various routes of inoculation, various doses of antigen, and several immunological tests in order to account for some of the variations found in studies on the serology of *M. tuberculosis*. The results presented in this paper indicate that the antibody response depends on the route of inoculation, the amount of antigen administered and the method of assay. The immunoglobulin associated with hemagglutination-lysis and hemagglutination is also characterized.

Material and methods. Antigens. Mycobacterial antigens were prepared from a strain of *M. tuberculosis* isolated in the Diagnostic Laboratory of the University of Iowa. This strain was pathogenic for guinea pigs, but was not pathogenic for New Zealand rabbits at the dose used in this study.

Organisms, cultured on Lowenstein-Jensen slants for three weeks, were suspended in Kirchner's Medium and dispersed by shaking in a vaccine bottle containing glass beads. Large particles were allowed to settle and the top suspension was withdrawn with a syringe. This suspension was diluted with Kirchner's Medium to a density equivalent to a McFarland tube #3.

Viable counts were obtained by placing 0.5 ml of 10-fold dilutions on Dubos Oleic-Albumin (Difco) plates. After incubation for 3 weeks at 37°C, the colonies were counted and approximated 10^6 ml.

Heat-killed tubercle bacilli were prepared by submerging a vaccine bottle containing the suspension of live tubercle bacilli in a 60°C water bath for 45 minutes.

Phenol-killed tubercle bacilli were prepared by the addition of 0.5 g phenol crystals to 10 ml of a suspension of tubercle bacilli. After incubation at 37°C for 24 hours, the bacilli were washed and resuspended in Kirchner's Medium. Viability tests were performed on all suspensions of killed tubercle bacilli.

Inoculation of animals and collection of serum. Guinea pigs (400-600 g) obtained from a local breeder were used for antibody production. 1 ml of antigen was injected into each guinea pig intracardially (i.c.), intraperitoneally (i.p.) or subcutaneously (s.c.) into the inguinal area. 2 ml of blood

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were obtained by cardiac puncture prior to antigen inoculation to obtain control serum, and thereafter at weekly intervals. The blood from each experimental group was pooled and frozen at −20°C.

Antibody assay. Hemagglutination (HA) and hemagglutination lysis (HL) tests were performed according to the drop modification of the Middlebrook-Dubos test(17). Old Tuberculin (Lederle) was concentrated 4-fold the international standard by the manufacturer.

Sera were tested for the presence of precipitating antibody by the agar double diffusion precipitin (DDP) test(18). The test antigen was a culture filtrate of the H37Ra strain of *M. tuberculosis* (Difco). The precipitin tests were incubated at 37°C for two weeks, with daily observations.

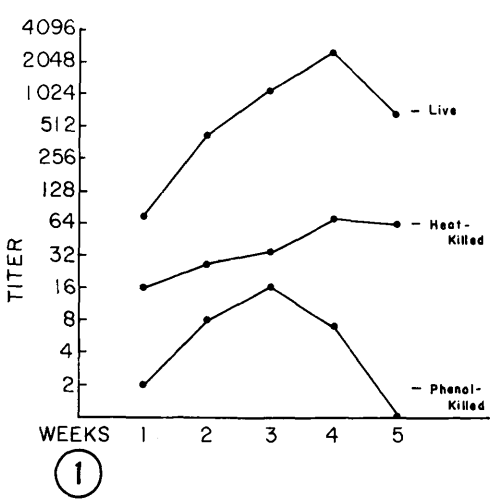
Rabbits and guinea pigs were tested for the presence of antibody which would demonstrate passive cutaneous anaphylaxis (PCA). Guinea pigs were injected i.c., i.p., or s.c. with a viable suspension of tubercle bacilli and the test sera were collected at 1-5 week intervals. The test serum from rabbits injected i.v. was collected 5 weeks after inoculation. 1/10th ml of test serum was injected intradermally into the shaved abdomen of white guinea pigs. No more than 6 serum specimens were injected per guinea pig. 1 hour after inoculation of the test sera, a mixture of 0.25 ml of Old Tuberculin (Eli Lilly) plus 0.25 ml of 0.5% Evans' Blue dye solution was injected i.c. into the guinea pigs. The animals were observed at frequent intervals for 8 hours for the appearance of dye at the site of serum injection.

Preparation of antiserum for immunoglobulin study. 12-day cultures of *M. tuberculosis* ($H_{37}Rv$) grown in Kirchner's medium with Tween 80 were diluted with Kirchner's medium to give an optical density representing 1×10^7 viable *M. tuberculosis* per ml.

Male albino guinea pigs were injected intracardially with 0.2 ml of the suspension of organisms. Serum was obtained 3 weeks after infection.

Column chromatography. Separation of 19S and 7S globulin was accomplished by the technique of Möller(19). Sephadex G-200 was hydrated in 0.15 M phosphate buffered NaCl pH 7.3. 2 ml of serum were applied to the column and 3 ml fractions were collected. The protein concentration of each fraction was measured by adsorption at 280 mμ.

Immunoelectrophoresis. LKB 6800 A immunoelectrophoresis equipment (LKB Pro-



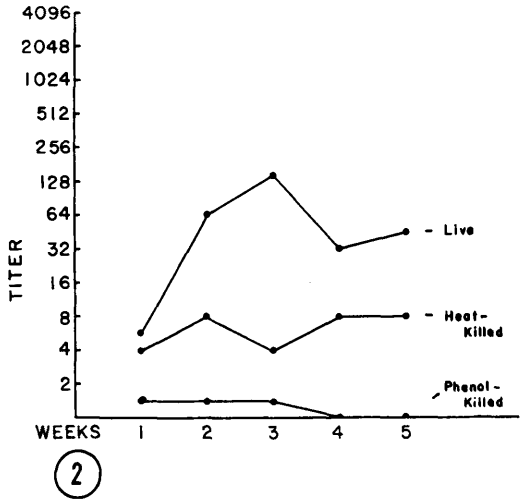


FIG. 1. HL antibody response in rabbits inoculated intravenously with live, heat-killed, and phenol-killed *M. tuberculosis.*

FIG. 2. HA antibody response in rabbits inoculated intravenously with live, heat-killed, and phenol-killed *M. tuberculosis.*

dukter Ab, Stockholm, Sweden) was used. The procedure recommended by the manufacturer as described in the operating manual was followed. All antisera used for developing precipitin bands were obtained from Immunology Inc., Glen Ellyn, Illinois.

Results. Antibody response in rabbits. To determine the antibody response to various preparations of *M. tuberculosis,* live, phenol-killed, and heat-killed tubercle bacilli were inoculated i.v. into rabbits. The HL antibody titers obtained in these rabbits are shown in Fig. 1., and the HA antibody titers in Fig. 2. Inoculation of live bacilli resulted in the highest HA and HL antibody titers, whereas the titers obtained with phenol-killed organisms were the lowest.

Antibody response in guinea pigs. Live or phenol-killed preparations of *M. tuberculosis* were injected i.c. and i.p. into groups of three guinea pigs. 6 guinea pigs were injected s.c. with live tubercle bacilli. The antibody titers obtained by the HL test are shown in Fig. 3.

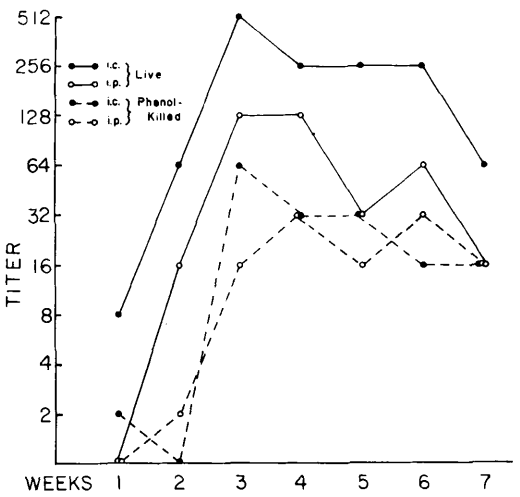


FIG. 3. HL antibody response in guinea pigs inoculated intracardially or intraperitoneally with live or phenol-killed *M. tuberculosis.*

Those guinea pigs which received live bacilli *via* the i.c. route of inoculation produced the highest HL titers. As with rabbits, the guinea pigs injected with phenol-killed organisms produced less HL antibody than those injected with live bacilli. Guinea pigs inoculated s.c. with the same dose of organisms did not produce antibodies detectable by the HL test. These results have been repeated in other experiments.

To determine the effect of dosage and type of serological test on antibody response 4 × 10^6, 3 × 10^5, and 3 × 10^3 viable units of tubercle bacilli were injected into guinea pigs by 3 routes: i.c., i.p., and s.c. Each group consisted of 4 guinea pigs. The HA and HL antibody titers of the sera collected from guinea pigs injected with a high dose (4 × 10^6 viable units) of tubercle bacilli are shown in Fig. 4. The highest HA and HL antibody

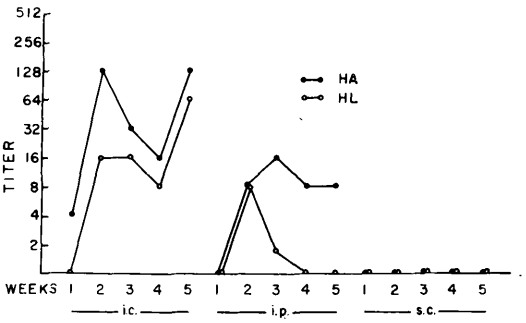


FIG. 4. HA and HL antibody response in guinea pigs inoculated intracardially, intraperitoneally, or subcutaneously with live *M. tuberculosis.*

titers were found in those animals injected i.c. Animals inoculated s.c. produced neither HA nor HL antibody. HA and HL antibody was not found in the sera of any of the guinea pigs injected with 3 × 10^5 or 3 × 10^3 viable units of tubercle bacilli.

Additional antibody studies. Assay of guinea pig serum by the DDP test did not reveal the presence of precipitating antibody. Only sera from rabbits which had been given a booster inoculation of live tubercle bacilli contained precipitating antibody.

Antibody giving rise to PCA was not demonstrable in either rabbit or guinea pig sera.

Chromatography of guinea pig serum. Fig. 5 contains the results of gel filtration studies. Most of the HL activity and all of the HA activity was present in the 19S fraction (first peak). However, a small amount of HL activity (8 units) was found in the 7S fraction (second peak). The small amount of HL activity in the 7S peak is probably due to some retardation of 19S immunoglobulin in the column. No antibody activity was found using unsensitized sheep erythrocytes.

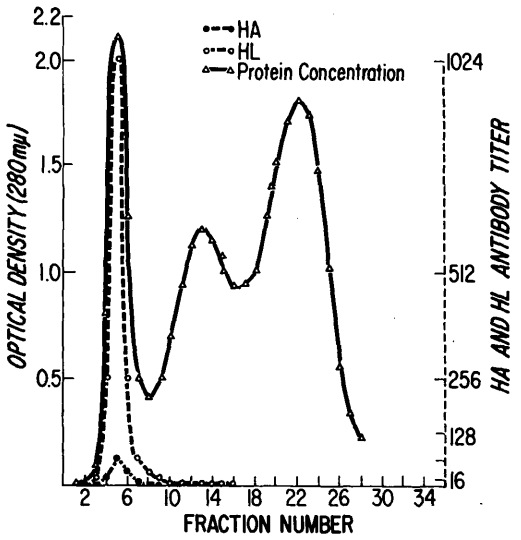


FIG. 5. Separation of *M tuberculosis* antibody activity from guinea pig serum by gel filtration.

Treatment of the antiserum with an equal volume of 0.2 M 2-mercaptoethanol (2-ME) followed by dialysis in phosphate buffered NaCl containing 0.02 M iodoacetamide resulted in complete loss of antibody activity (Table I). This would indicate reduction of the 19S immunoglobulin into inactive subunits.

TABLE I. Antibody Activity of Guinea Pig Serum After 2-Mercaptoethanol Treatment and Dialysis.

Sample	HL units*	HA units†
Serum + buffer	512	128
Serum + 2-mercaptoethanol	<2	<2
Buffer + 2-mercaptoethanol	<2	<2

* Hemagglutination lysis.
† Hemagglutination.

Immunoelectrophoretic studies. The 19S and 7S peak fractions obtained by chromatography were precipitated with ammonium sulfate and redissolved to give a concentration of 8-fold and 13-fold, respectively. Immunoelectrophoretic studies on these concentrated fractions using rabbit antiserum to guinea pig total serum globulin (top slide) and 7S globulin (bottom slide) are shown in Fig. 6. A 19S precipitin band to the right of the well containing the 19S fraction and the absence of a 7S precipitin band indicates the homogeneity of the 19S fraction (top slide). The bottom well containing the 7S fraction gave a 7S precipitin band and a faint band which could be 19S antibody. However, it appears to be closer to the trough than the 19S precipitin band near the top well. Fig. 7 shows the effect of 2-ME on the 19S and 7S fractions. An increase in the diffusion rate of the 19S antibody, resulting in formation of a precipitin band closer to the trough than observed with untreated 19S antibody, indicates reduction to subunits (top slide). 2-ME treatment of the 7S concentrated fraction had no effect on the 7S precipitin band (bottom slide).

Discussion. That the route of inoculation does have an effect on antibody response in guinea pigs is demonstrated by production of HA and HL antibody in animals injected either intracardially or intraperitoneally, but not by animals injected subcutaneously.

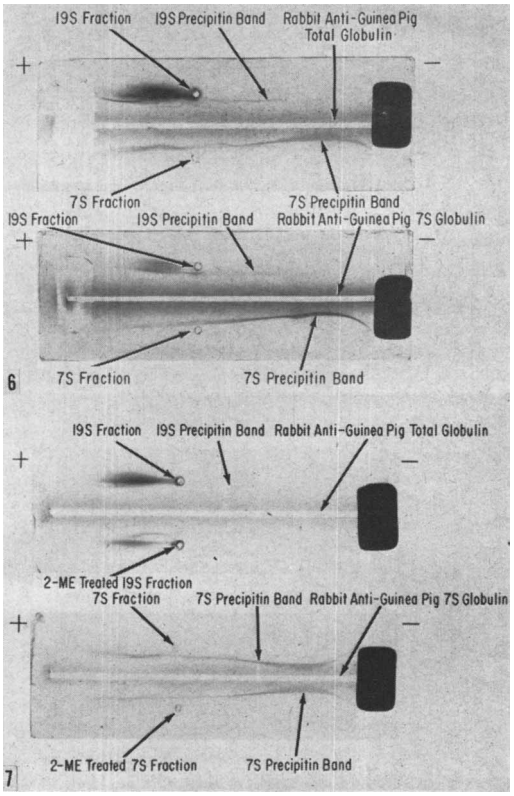


FIG. 6. Immunoelectrophoresis of guinea pig 19S and 7S concentrated fractions obtained by gel filtration.

FIG. 7. Immunoelectrophoresis of 2-mercaptoethanol treated and untreated 19S and 7S guinea pig globulin.

Guinea pigs with intracardially injected live tubercle bacilli produced higher HL and HA antibody titers than did intraperitoneally injected animals. Antibody to live tubercle bacilli in guinea pigs could not be demonstrated by use of the PCA technique, nor did the DDP test demonstrate precipitating antibody following primary injection. The absence of correlation between the precipitin and hemagglutination titers in certain antisera has also been shown by Richter(20).

The dose of *M. tuberculosis* is an important factor in determining antibody response. Guinea pigs injected i.c. or i.p. with 10^6 viable units of *M. tuberculosis* did produce HA and HL antibody, whereas animals injected with lower doses did not produce detectable antibody.

A possible explanation for the lack of antibody in guinea pigs injected s.c. may be the means by which the tubercle bacilli are handled in the body. Cohn(21) reported that *Escherichia coli*, after residing in polymorphonuclear leukocytes, are less antigenic than organisms that have resided in macrophages. Pavillard(22) has shown that alveolar macrophages are less efficient than peritoneal macrophages in killing *E. coli* and *Staphylococcus aureus in vitro*. Alveolar macrophages contain higher levels of acid phosphatase and lysozyme indicating physiological differences in reticuloendothelial cells from various organs within the same animal.

Studies with a dye-protein complex show that after intravenous and intraperitoneal injection, the dye-protein was spread throughout the body whereas, the dye was limited to local macrophages and lymph nodes after intradermal or subcutaneous injection(23). The lack of HA and HL antibody in guinea pigs inoculated subcutaneously with tubercle bacilli could possibly be accounted for by a faster destruction or alteration of their antigenic components when entrapped in the inguinal lymphatics as opposed to other areas of the host.

These studies show that the antibody responsible for hemagglutination (HA) and hemagglutination lysis (HL) of old tuberculin coated erythrocytes is primarily 19S immunoglobulin. A small amount of HL antibody was found in the 7S fraction obtained by gel filtration. However, treatment of whole guinea pig serum with 2-mercaptoethanol inactivated the antibody.

Studies similar to those reported here have been done by Daniel(24,25) who has shown that injection of soluble old tuberculin or alum precipitated purified protein derivative elicited 19S antibody production. Injection of old tuberculin in adjuvant, killed or live *M. tuberculosis* resulted in production of both 19S and 7S antibody. Turcotte *et al*(26) found both 19S and 7S antibodies in patients with tuberculosis. Patients with active disease had low levels of 19S antibody but high levels of 7S antibody. Both of the above workers feel that 7S antibody is associated with injection of live organisms or the active form of tuberculosis.

The major difference between the results in this paper and those of Daniel and Turcotte *et al* is in the absence of 2-ME resistant 7S antibody. However, this could result from differences in the host, dose of microorganism and time at which serum was obtained from the animal. The $H_{37}R_v$ strain of *M. tuberculosis* used in our studies is virulent for guinea pigs and therefore should have evoked a 7S antibody response.

Kim, Bradley and Watson(27) have recently reported that the first immunoglobulin synthesized following primary immunization of piglets with actinophage is a 19S γ G immunoglobulin. Whether the immunoglobulin responsible for hemagglutination was a 19S γ G or 19S γ M would require further detailed investigations.

Summary. Live *Mycobacterium tuberculosis* injected intravenously into rabbits produced higher titers of hemagglutination (HA) and hemagglutination lysis (HL) antibodies than were obtained using phenol-killed or heat-killed *M. tuberculosis*. In guinea pigs, injection of 10^6 cells of *M. tuberculosis*, either intracardially or intraperitoneally, resulted in HA or HL antibody, whereas antibody was not produced by animals injected with lower doses. HA or HL antibody was not produced by subcutaneous injection of live organisms.

Serum obtained from guinea pigs three

weeks after intracardial injection of *M. tuberculosis* strain $H_{37}R_v$ was used to determine the immunoglobulin class of antibody responsible for the HA and HL reaction. Only 19S immunoglobulin could be associated with this antibody activity.

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Studies on Hemagglutination by Rubella Virus.* (32560)

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The discovery by Stewart *et al*(1) of an hemagglutinin which appeared in BHK21 cultures after infection began a new period in the immunological study of rubella: This discovery was based on the removal of an hemagglutinin-inhibitor both from the fetal calf serum used to grow the virus and from the sera to be tested for specific hemagglutinin-inhibiting antibody.

Recently we reported the preparation of hemagglutinin (HA) from infected BHK21 culture fluids(2) by treatment of tissue culture fluids with sodium ethylenediaminetetraacetate (EDTA), which separates HA from inhibitor. We presented evidence for the hypothesis that the attachment between HA and inhibitor depended on the presence of divalent cations and that EDTA released inhibitor by its binding action on cations.

In this paper, we report a more detailed study of rubella virus (RV) HA prepared by this method.

Materials and methods. Virus: The RA 27/3 strain(3) in the 18th to 24th passage

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