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The Effect of Immune IgM and IgG on the Infectivity of Trichinella Spiralis in Mice

Clare K. Kwan

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LOMA LINDA UNIVERSITY

Graduate School

THE EFFECT OF IMMUNE IgM AND IgG ON THE INFECTIVITY OF *TRICHINELLA SPIRALIS* IN MICE

by

Clare K. Kwan

A Dissertation in Partial Fulfillment of the

Requirements for the Degree

Doctor of Philosophy in the Field of Microbiology

August 1970

I certify that I have read this dissertation and that in my opinion it is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Dedicated to my parents,

Capt. and Mrs. P. L. Kwan

and

to my American grandmother,

Mrs. Ida H. Gjording

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INTRODUCTION

Acquired immunity is protection against microbial diseases which is established in an individual either passively or actively at any time after birth.

Recovery from diseases caused by bacterial or viral infections very commonly carries with it a decreased susceptibility to subsequent attack by the same organism. This decreased susceptibility is attributed to antibodies produced within the host, which are either circulating in the blood stream or are fixed to the tissue cells, or both. These antibodies are specific and protective in nature and can be usually measured by serological tests to a certain degree, such as, by agglutination, complement fixation, precipitation, immobilization, direct and indirect fluorescent antibody techniques and many others.

When a non-immunized person suffers a wound with the consequent risk of the development of tetanus, a useful way to immediately protect him against this risk is to immunize him passively with tetanus antitoxin produced in man or other mammals. This passive immunity is short-lived, lasting approximately six weeks.

In helminth infection, the picture is more complex, due to the larger assortment of antigens a worm possesses; as may be present in the cuticle, tissues, metabolites, and antigens associated with

the various larval stages before the organism reaches the adult stage.

The complexity of helminth antigens renders the isolation of an antigen, or a group of antigens that are responsible for protective immunity, difficult. Parasitologist-immunologists have called these protective antigens "functional antigens," which in turn cause the production of "functional antibodies."

Review of the Literature

In surveying the literature, one notices that a considerable amount of work has been done in various aspects of immunity to protozoa as well as metazoa.

Oliver-Gonzalez (1941) was one of the first to demonstrate that there are at least two sets of antigens in Trichinella spiralis (Owens, 1835) Railliet, 1895, namely, the adult and the larva. With the usage of modern tools in research, Kagan (1967) and his group have detected 12 to 16 antigens from T. spiralis larvae by immunoelectrophoresis. By means of DEAE Sephadex chromatography, Kent (1963 a, b) isolated nine protein fractions from T. spiralis, five from Ascaris lumbricoides and eight from Echinococcus granulosus. Certain of these fractions could elicit a hypersensitivity response to a certain degree when injected intradermally into humans who had been previously infected by the specific organisms. Chordi and Kagan (1965)

isolated nineteen antigens by immunoelectrophoresis from the sheep hydatid fluid. Ten of these nineteen antigens were of parasite origin.

Cohen (1961) prepared a gamma globulin fraction from the pooled sera of persons with chronic malaria. When this was injected into children with malaria, a reduction in trophozoites and clinical signs occurred. Rose (1963) and Long and Rose (1965) worked with passive transfer of resistance to Eimeria tenella in fowls by means of immune globulin. Challenging infection was given four and one-half hours after the repeated intravenous and intraperitoneal injections of the immune globulin. The experiment was not successful when the challenging infection of oocysts was given orally or intrarectally. However, when the challenging infection of 0.84×10^6 sporozoites was given intravenously, the transferred globulin did give protection. The oocyst number was reduced to 2.6×10^5 as compared with the control (injected with normal globulin) for which the yield was 82.5×10^5. By using parabiotic mice, Crandall (1965) demonstrated the transfer of immunity from an actively immunized partner to a non-immune one. Female mice were immunized orally with 20,000 to 40,000 eggs of Ascaris suum per mouse, three to four times in two week intervals. A high level of resistance was obtained in this mouse strain as measured by the number of lung larvae recovered eight days after a challenge infection as compared to the challenged normal, previously

uninfected mice. One month after the last immunization the mice were parabiosed. In one pair, one mouse was immune and the other normal, and in the other pair, both were non-immune. Ten days after the union, the non-immune member of the first pair and one of the mice of the second pair were challenged with 12,000 embryonated eggs by mouth. Eight days after this challenge, all the mice were killed. The results showed a significant reduction in worm burden in the non-immune partner of the first pair. There was little crossing over of the larvae from the challenged partner to the unchallenged mouse in both pairs. Zaiman (1953) reported that when one member of the parabiosed pair of rats was given a known number of Trichinella spiralis larvae in a single dose, that rat became resistant to subsequent challenge one month later. The resistance of the uninfected rat was less than that of its infected mate, but greater than that of the paired, uninfected, control rats. A total of 5,975 T. spiralis larvae was given per os to various single members of the experimental pairs. One month later, these members were again challenged with a total of 5,957 T. spiralis larvae. At the same time this same number of larvae was given to the uninfected members of the pairs, and 5,704 larvae to the controls. Five days later all the rats were sacrificed for the recovery of adult worms in the intestine. The result showed that a total of 110 adult worms was recovered from the

members that received the challenge infection, 3,280 adult worms from the controls and only 1,280 from the "uninfected" members of the previously infected single partner. Zaiman concluded that there was a transfer of antigens as well as antibodies from the immunized rats to their parabiotic mates via the common blood circulation.

Immunity in rats against T. spiralis was first observed by Ducas (1921). This immunity was further investigated by Culbertson and Kaplan (1937), who found that in rabbits it was humoral in nature and could be passively transferred. Later, Culbertson (1942) repeated this experiment with rats. He used immune rat serum and transferred it to uninfected rats. A challenge infection of 250 larvae was given. He found a five-fold decrease (60 vs 11.2) in the number of adult worms in the intestinal tract of the rats which received the immune serum, as compared to the rats receiving normal serum. There was a four-fold decrease (47,000 vs 12,000) in the larva count of the two groups. Dorin (1946) collected immune rabbit serum two weeks after injecting the rabbits with 400,000 T. spiralis larva homogenate suspended in aluminum cream, he then injected 9 ml of immune rabbit serum into rats, and normal rabbit serum into a group of control rats. He observed that eleven days following a challenging infection of 4,500 larvae per rat, the animals that received immune rabbit serum were clean looking, sleek and active, while the ones that received the normal

rabbit serum possessed bloody-red noses and were moribund, indicating that the immune serum protected the animals from the adverse effects of the parasite. Hendricks (1953) collected hyperimmune serum from mice which were given three stimulating T. spiralis infections at three-week intervals. When six-week-old mice were injected with this hyperimmune serum, and then challenged with 200 larvae, they yielded approximately one-half as many adult worms as from the controls which were given normal mouse serum (52.2 vs 140.6).

Mauss (1941) demonstrated that the protective antibody against T. spiralis was associated with the euglobulin of the serum. After incubating the larvae in vitro with immune rabbit serum, immune euglobulin, normal serum and normal euglobulin for 18 hours, they were fed to rats. Seventy-two hours later, one-third as many adult worms were recovered from rats that were given the larvae previously incubated in immune serum than from rats given larvae incubated in normal serum. There was a five-fold decrease in adult recoveries from the larvae incubated in immune euglobulin as compared to larvae incubated in normal euglobulin. Similar findings that the protective action against T. spiralis is contained in the globulin of the serum was later obtained by Komandarev (1966).

Kagan et al (1968) fractionated two serum samples from human trichinosis by Sephadex G-200, and found that the IgM was more re-

active with the bentonite flocculation test (1:320) and the IgG was less reactive (1:20). The titer of the whole serum was 1:1280. This difference was explained on the basis that the serum was collected during the acute phase of the illness when the majority of the antibody would be associated with the IgM, and the production of IgG had not reached a peak.

Crandall et al (1967), using the indirect fluorescent antibody technique, demonstrated that there was a relative increase in the IgM-containing cells in the intestinal tissue, reaching a peak at ten days after infection with 10,000 T. spiralis in rabbits. IgG-containing cells reached a peak at 30 days, gradually decreased and again peaked at 8 days after the second challenge infection of 12,000 larvae. The relative increase in the number of the IgM-containing cells in the spleen and popliteal lymph nodes began after the 14th day post infection and continued through the 9th day after the second challenge infection. There was no increase in the number of the IgG-containing cells. There was no change in the relative number of the IgA-containing cells in either the intestine or the spleen and lymph nodes. In working with humoral antibodies against T. spiralis in rabbits, Crandall and Moore (1968) showed a rise in titer in the IgM fraction beginning at three or four weeks after the immunizing dose, depending on the individual rabbit. The IgM antibodies reached a peak between four to six weeks,

and there was a rise in the IgG antibodies, beginning at the 5th week postinfection. However, the duration of IgM in the circulation has not yet been determined.

DiConza (1969) showed that when the serum collected from mice one week after an oral infection of Hymenolepis nana eggs was given to uninfected mice, it prevented the majority of the larvae from developing. The active material for protection is found in the IgG. The average larval count of H. nana in the intestine of the mice that received immune IgG was 14, and that of the mice that received normal IgG averaged 63. It was also shown that immune IgM had very little protective effect (46 vs 51).

Objective of the Study

Many workers have demonstrated that the immunity against tissue-inhabiting parasites is in part humoral in nature and can be transferred from host to host. It is the purpose of this study to demonstrate the effect of the immune serum fractions, namely IgM and IgG, on the infectivity of Trichinella spiralis, as compared to the effect of the normal serum, normal fractions and immune whole serum. In order to minimize the variables of the antigen-antibody combination in vivo, the larvae were to be preincubated in vitro with the sera and serum fractions before infecting the mice. This would

show which fraction of immune globulins is responsible for passive protective immunity against T. spiralis infection, and its direct effect upon the parasite, especially since one of the immunoglobulins or both will give reduced infectivity of the larvae.

The criteria used in determining whether preincubation with the sera and serum fractions affect the infectivity of the larvae are:

1. The semiquantitative amount of precipitate found in the medium in which the larvae have been incubated, and precipitate afixed to the larvae.

2. The number of adult worms recovered from the intestine of the mice 72 hours after gavaging them with the preincubated larvae.

3. The number of larvae recovered from the musculature of the mice 28 days after gavaging them with the preincubated larvae.

MATERIALS AND METHODS

Preparation of Trichinella spiralis Larvae for Experimental Use

The larvae were obtained from infected stock rats maintained in the Microbiology Department, Loma Linda University. Larvae were digested from the musculature of the rats by use of artificial aqueous digestive juice consisting of 0.6% of 1:10,000 pepsin and 0.8% HCl incubated at 37°C for six hours (Kwan et al, 1965).

Preparation of Immune Serum

Four New Zealand female rabbits, weighing 2 kg each were initially gavaged with 10,000 larvae each by intragastric intubation with a 0.5 x 44 cm nasogastric tube. In order to facilitate passage of the tube into the stomach and aspiration of gastric juice to insure the location of the tube, food but not water was withheld from the animals for eight hours before gavaging. Samples of blood were obtained from the marginal ear vein before gavaging, in order to establish a base-line anti-Trichinella titer. The second blood sample was taken by the same method 21 days after the initial gavage for the purpose of determining the primary antibody response. On the same day after each bleeding, the rabbits were again given the larvae. These rabbits were bled by cardiac puncture at 14 days after the second gavage of 12,000 larvae and at 21 days after the third gavage of 15,000 larvae

(Figure 1). Samples of serum were separated from clots by centrifugation and stored at -20°C until used.

Preparation of Crude Saline Extract of Trichinella spiralis for in vitro Detection of Antibody Titer

Larvae were extracted with approximately twenty times their volume of pyridine to remove the polysaccharide coating, thus to "unmask" the antigenic architecture of the organism (Labzoffsky et al, 1959). The larvae were then washed four times with distilled water. These larvae were resuspended in four times the volume of 0.85% NaCl, pH 7.0, and ground with a tissue homogenizer until all the larvae had been cut into several fragments, as revealed by microscopic examination (Melcher, 1943, Kagan, 1960). To this mixture five times the volume of saline was added and allowed to stand for three hours at room temperature and 12 hours at 4°C. The mixture was then centrifuged at 10,000 x g for 30 minutes and the sediment discarded. The supernatant solution was heated at 56°C for one hour, and centrifuged again for 30 minutes at 10,000 x g. The supernatant antigen solution was removed and merthiolate (Thimerosal powder, Eli Lilly and Co., Indianapolis, Indiana, U. S. A.) added to a final concentration of 1:10,000, titrated for 100% unit hemolysis against immune rabbit serum for the optimum amount of antigen (Lenette, 1964)

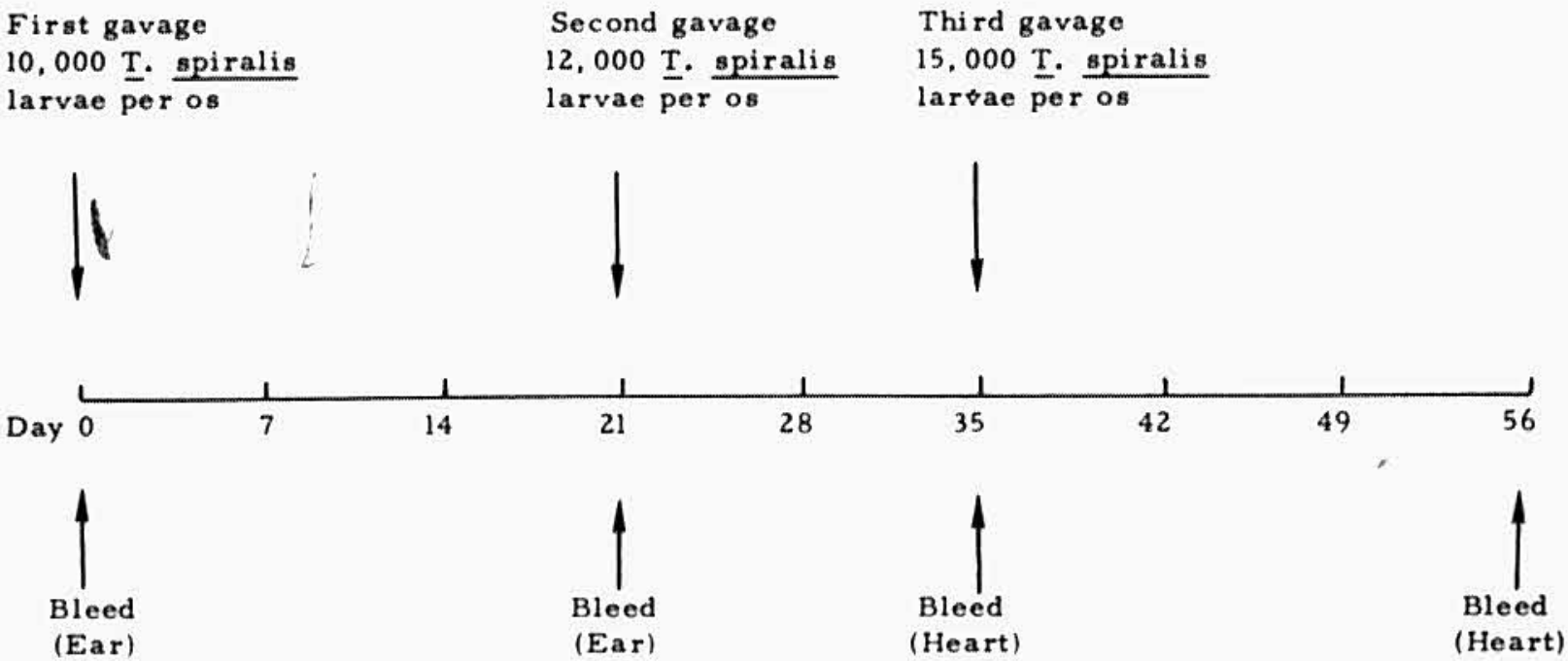


Figure 1. Schedule of Gavaging and Bleeding of Rabbits

and stored at -20°C until used.

Fractionation of Sera

Sera were precipitated with methanol at -6°C (Dubert et al, 1953). The gamma globulin precipitate was then fractionated by gel filtration using a column of 2.5 cm x 90 cm packed with Sephadex G-200. The eluent was 0.1 M Tris-HCl, pH 8.0 in 0.2 M NaCl (Flodin and Killander, 1962). Fractionation was carried out at 2°C. The optical density of each effluent portion was determined by a Beckman DBG Spectrophotometer, using a wavelength of 280 mμ. Two peaks were observed.

The effluents under the two peaks were pooled separately for each peak. They were concentrated by pervaporation, dialyzed against 0.85% NaCl and the protein content determined by the Lowry's test (Lowry et al, 1951). Immunoelectrophoresis was performed on the concentrated, dialyzed fractions according to Scheidegger's method (Scheidegger, 1955), using a commercially prepared anti-rabbit serum as the precipitating antibodies in the trough (Hyland, Division Travenol Laboratories, Inc., Los Angeles, California, U. S. A.).

Antibody Titers determined by the Complement Fixation and Passive Hemagglutination Tests

Antibody titer of whole serum and fractions were performed by testing the two-fold serial dilutions by complement fixation (Lenette, 1964). Anti-sheep hemolysin, guinea pig complement were titrated for 100% unit of hemolysis with 2% sheep red blood cell suspension in a total volume of 1.0 ml. In the test two units of the guinea pig complement and two units of antigen in 0.2 ml volume were used. Serum controls and reagent controls, such as, complement control for antigen, hemolytic control, sheep red blood cell control and non-specific antigen control (using antigen from previous lot) were included in each set of determinations. For the test, 0.2 ml of serum, serum dilution, fraction or fraction dilution, 0.2 ml antigen, 0.2 ml complement were incubated at 4°C overnight. The test were completed at 37°C by the addition of 0.4 ml of sensitized cells, consisting of equal portions of hemolysin and 2% sheep red blood cells. When no hemolysis was observed, the complement fixation was considered positive, if all controls were proper.

The passive hemagglutination test by the method of Campbell et al (1964) was done on the IgM and IgG serum fractions. The same crude saline extract of the trichina larvae was the antigen, and tanned sheep red blood cells were used. A block titration was done on each

lot of antigen to establish the optimal dilution of antigen for coating the tanned cells with an immune serum sample. The tests were read two hours and 24 hours after incubation at room temperature.

Reaction of Serum and Fractions with Live Trichinella spiralis Larvae

Five hundred viable, washed muscle-free T. spiralis larvae were incubated with 0.3 ml of serum or fractions at 37°C in a water bath for eighteen hours by the method of Mauss (1941). The incubated suspension was placed on a slide and examined microscopically for the presence of precipitate in the medium and around the oral orifice of the larvae. To document the fact that the observable precipitate was the end product of an antigen (larva) and antibody (serum or fractions) reaction, the indirect fluorescent antibody technique as per the method of Taffs and Voller (1962) was employed. The reagent used was fluorecein isothiocyanate conjugated 7S fraction of antirabbit antiserum (Cappel Laboratories, Inc., Downingtown, Pa). After incubation with serum or fractions, the larvae were washed with 0.01 M phosphate buffer, pH 7.1, and fixed with 3 ml of methanol for 10 minutes. The fixed material was washed thoroughly with buffer, and stained with the fluorescein-labelled antiserum for 30 minutes. After washing several additional times, the larvae were placed on slides under a coverslip and examined microscopically, using a AO Spencer micro-

scope with a fluorolume illuminator, and primary BG-12 filters and a OG-1 filter in the eyepiece.

Test for the Infectivity of the Incubated Larvae in Mice

Twenty-one-day-old weanling Swiss-Webster mice weighing 13 to 20 grams were randomly arranged in groups of 60 for experimentals and 60 for controls. The control animals were subdivided into 3 groups of 20: one group received larvae that had been previously incubated for 18 hours in normal rabbit serum, the second group received larvae that had been incubated in normal rabbit IgM and the third group was given larvae which had been incubated in normal rabbit IgG. The experimental animals were also subdivided into three groups of 20: one group received larvae which had been incubated in immune rabbit whole serum, one group received larvae that had been incubated in immune rabbit IgM and the third group was given larvae that had been incubated in immune rabbit IgG.

Each mouse was given 100 muscle-free viable larvae previously incubated in one of the above specified procedures after being washed three times with 0.85% NaCl. The gavage was accomplished by the use of a blunt, 18 gauge needle and a tuberculin syringe. The total volume given to each mouse was 0.1 ml.

Seventy-two hours post-gavage, half of the number of animals

of all the groups were sacrificed for the recovery of adult worms. The intestines were freed, slit longitudinally, and incubated in 0.1% NaOH at 4°C for 12 hours (Larsh and Kent, 1949).

Twenty-eight days post-gavage, the remaining mice were sacrificed for the recovery of larvae from the musculature by pepsin digestion.

The data obtained were submitted to statistical analyses using the square root transformation and calculating the t value (Steel and Torrie, 1960),

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S_p\sqrt{1/N_1 + 1/N_2}}$$

$$S_p = \sqrt{\frac{(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2}{N_1 + N_2 - 2}}$$

Where

$\bar{x}$ = mean

N = number of animals per group

S^2 = variance

RESULTS

In vitro Reaction of Serum and Fractions with Trichinella spiralis Larvae

Incubation of T. spiralis larvae with heat inactivated (56°C for 30 minutes) immune rabbit whole serum and immune IgG showed a 4+ precipitate in the medium, while only a 2+ precipitate was observed in the medium of larvae incubated with immune IgM. A small percentage of larvae showed precipitate around the oral orifice (Figure 2). There was no precipitate observed in the reaction with normal whole serum, normal IgG and normal IgM (Table I). Indirect fluorescent antibody staining showed the precipitate in the medium and on the larvae to be a bright fluorescent green, while the normal serum and fractions showed no fluorescent precipitate in the medium, and the larvae were yellowish to greenish in color (Figures 3 to 6). These procedures were repeated several times to identify the precipitate to be of an antigen antibody nature.

Fractionation of Rabbit Serum and Identification of Peaks by Immunoelectrophoresis

The scanning of the effluent from the Sephadex G-200 column chromatography at 280 m_{μ} wavelength gave consistently

Table I. Amount of precipitate observed in the medium and number and percent of larvae showing oral precipitate when Trichinella spiralis larvae were incubated in normal whole serum, normal IgM, IgG, immune whole serum, immune IgM and IgG for 18 hours at 37°C

Medium Used	No. of Larvae Counted	No. of Larvae with Oral Ppt	Percent of Larvae with Oral Ppt	Ppt in Medium (1 to 4+)
Saline	18	0	0	0
Normal Whole Serum	25	0	0	0
Immune Whole Serum	13	8	60	4+
Normal IgM	11	0	0	0
Immune IgM	21	1	5	2+
Normal IgG	61	0	0	0
Immune IgG	32	20	60	4+

Figure 2. In vitro reaction of anti-Trichinella antibody with live trichina larvae for 18 hours at 37°C. Shown are precipitates around the oral orifice and in the medium.

Figure 3. Indirect fluorescent antibody staining on reaction of trichina larva and immune whole serum, and

Figure 4. Normal whole serum.

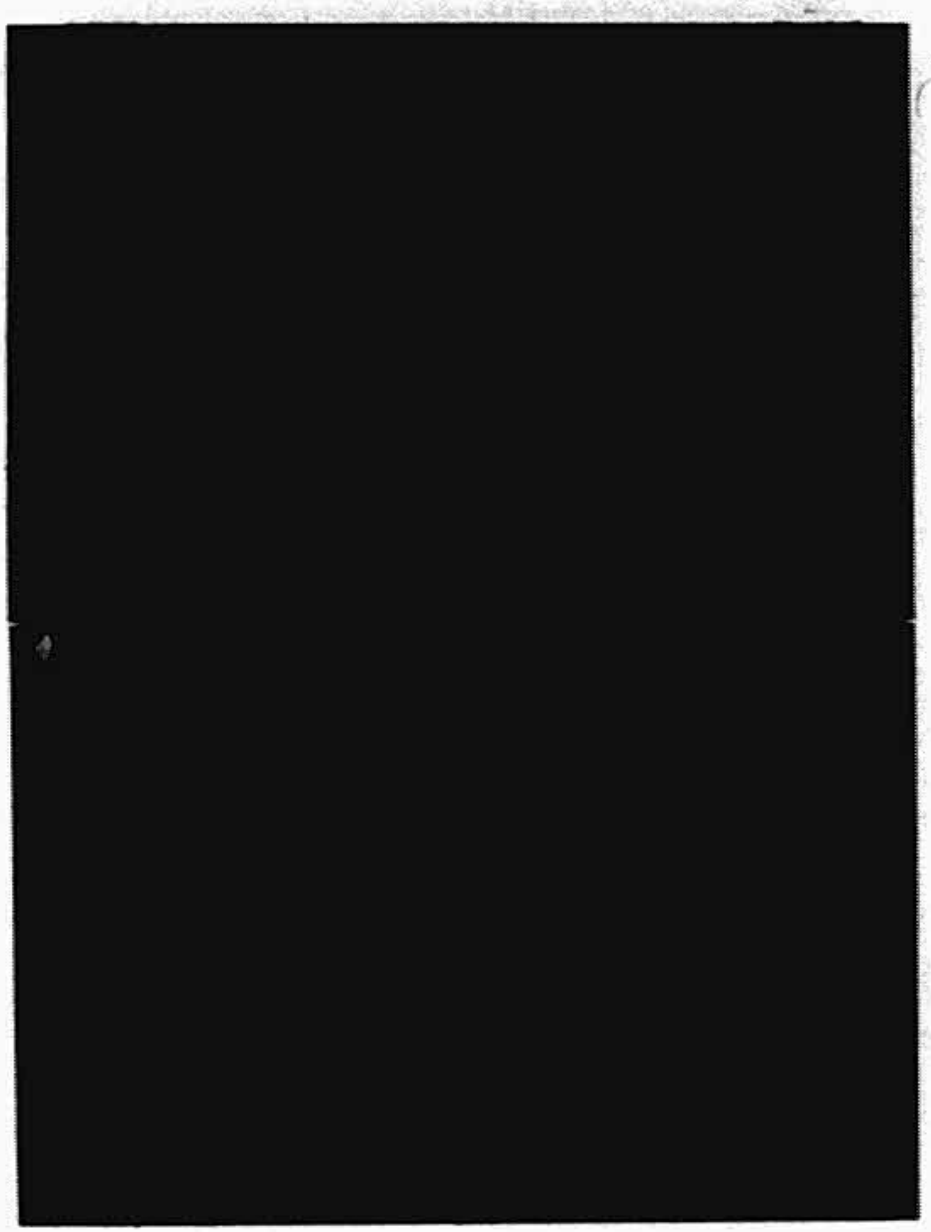


Figure 5. Indirect fluorescent antibody staining on reaction of trichina larva and immune IgM, and

Figure 6. IgG immunoglobulins.

two peaks per sample of the methanol precipitated rabbit gamma globulin (Figure 7). Fractions under each peak were pooled, concentrated by pervaporation, dialyzed against 0.85% NaCl and electrophoresed in thin layer agar electrophoresis in Veronal buffer, pH 8.2, with 4.2 mAmp/slide for two hours. A trough was cut in the agar after electrophoresis and 2 μl of antirabbit antiserum produced in goat (Hyland, Division Travenol Laboratories Inc.) was applied. This reaction showed the first peak to contain chiefly IgM with some lipoproteins, and the second peak was chiefly IgG (Figure 8 and 9). The above findings were confirmed by Hyland Laboratories by the use of their experimental antirabbit IgM and IgG antisera produced in goat as precipitating antibodies on immunoelectrophoresis. This is consistent with the theory of exclusion of the Sephadex G-200 column chromatography (Leach and O'Shea, 1965), that the molecules greater than a molecular weight of 200,000 do not penetrate the gel matrix, and are thus eluted first. The molecular weight of IgM is approximately 1×10^6 whereas that of IgG is approximately 1.5×10^5, which is eluted last.

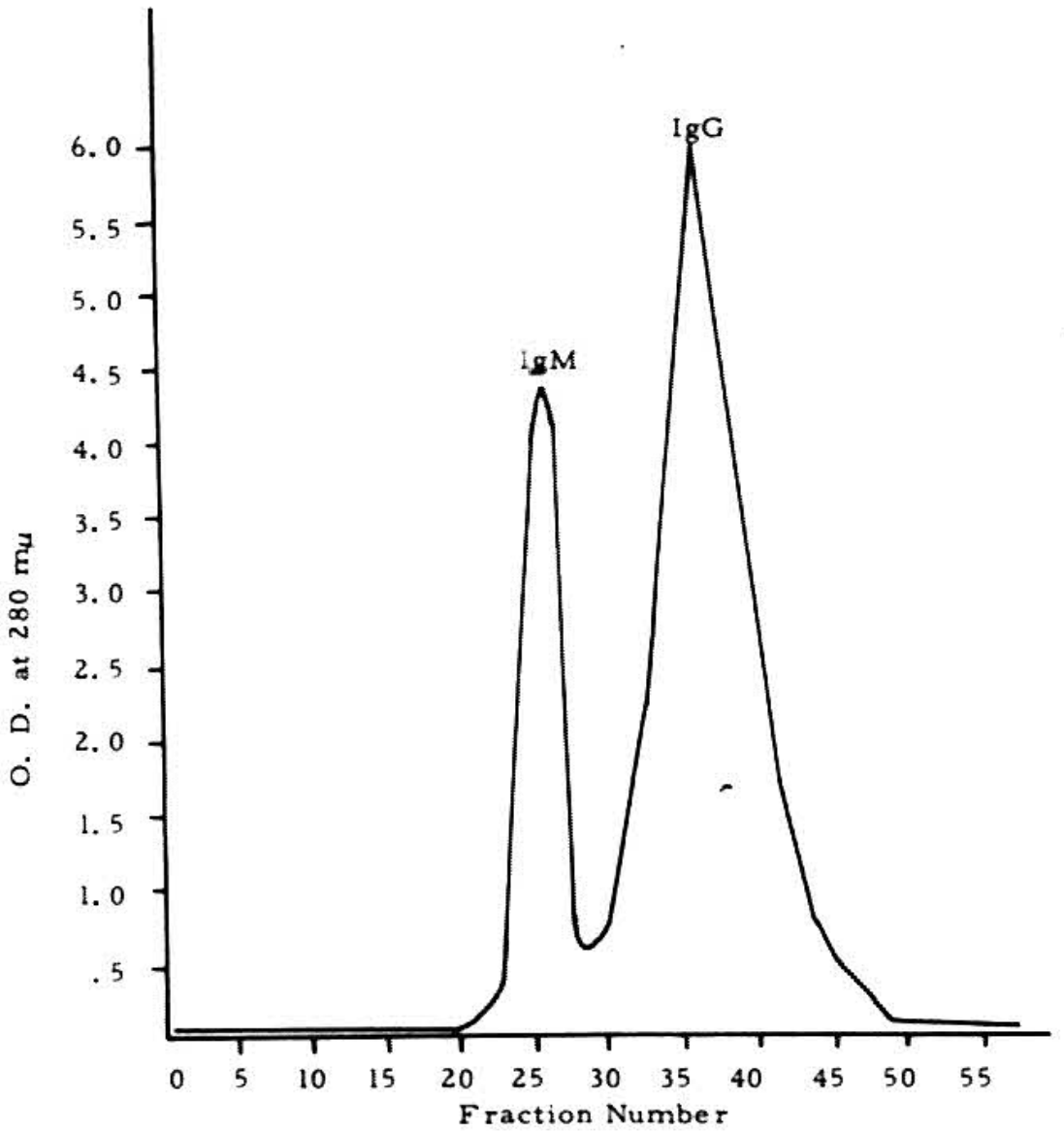


Figure 7. Elution profile of rabbit gamma globulin by Sephadex G-200 chromatography.

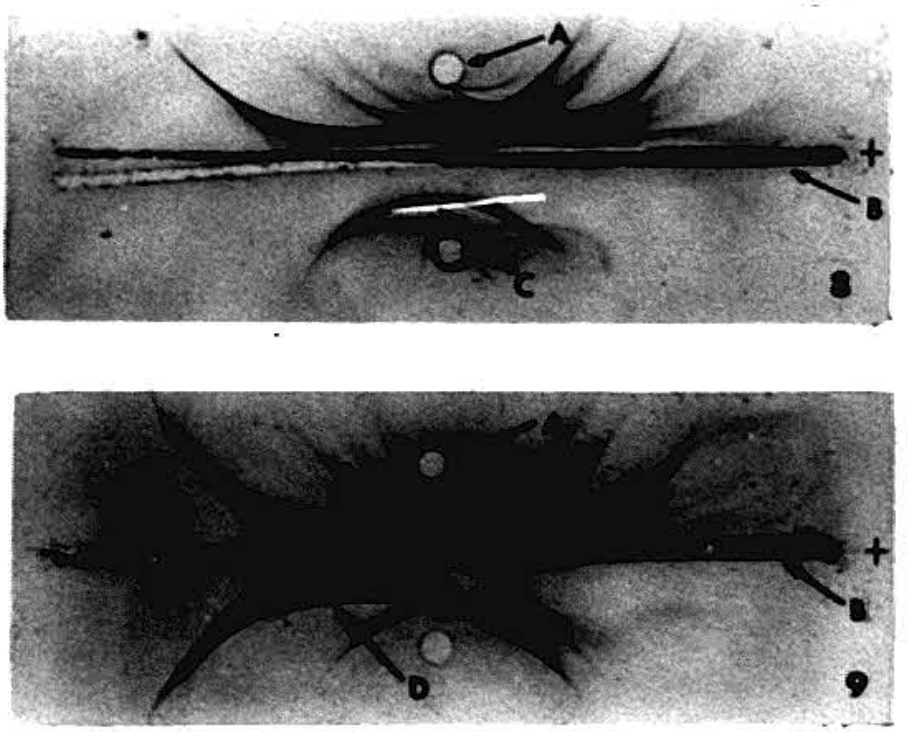


Figure 8. Immunoelectrophoretic pattern of rabbit whole serum and IgM, and

Figure 9. IgG.

A. Rabbit whole serum
B. Goat anti-rabbit antibody
C. IgM
D. IgG

Immune Response to Trichinella spiralis in Rabbits as measured by Complement Fixation Test

The rabbits were bled and challenged according to schedule (Figure 1). The antibody responses were approximately similar in all four rabbits as measured by the complement fixation test (Table II). Serum antibodies rose steadily after gavages, except for rabbit No. 3 which experienced some decrease in antibody titer after the last challenge. Complement fixation was also performed with the IgM and IgG fractions. Results showed that IgM and IgG titers were present concurrently, with the IgG higher than that of the IgM at all times (Figure 10). A decrease in titer of IgM was also observed in rabbit No. 3 at 21 days after the third challenge with 15,000 larvae.

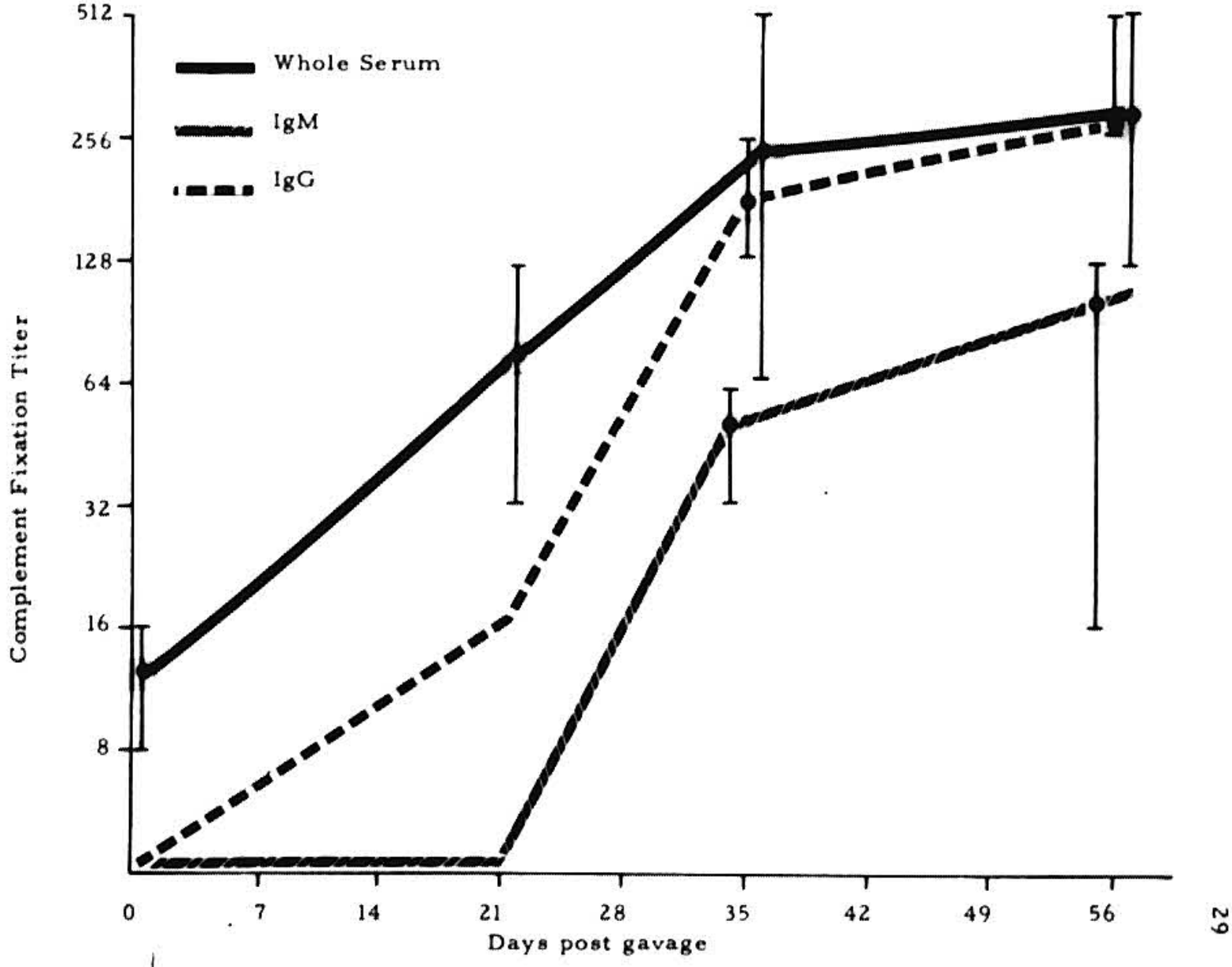
Immunoglobulin Response to Trichinella spiralis as Measured by Passive Hemagglutination Test

As determined by the passive hemagglutination test, there were no specific antibodies against T. spiralis in the IgM and IgG fractions from the rabbits before gavage (Table II and Figure 11). Twenty-one days after the first gavage, the IgM titer was 200, while the IgG fraction was zero. The serum collected by 14 days

Table II. Immune response of rabbits to Trichinella spiralis as measured by the complement fixation and passive hemagglutination tests.

	Rabbit Number	Complement Fixation Titer			Hemagglutination Titer	
		Whole Serum	IgM	IgG	IgM	IgG
Before gavage	1	16				
	2	8				
	3	8				
	4	16				
	Average	12	4	4	0	0
21 days after 1st gavage	1	64				
	2	32				
	3	128				
	4	64				
	Average	72	4	16	200	0
14 days after 2nd gavage	1	128	64	128	400	400
	2	64	64	256	200	800
	3	256	64	128	200	400
	4	512	32	256	200	800
	Average	240	56	192	250	600
21 days after 3rd gavage	1	256	128	256	200	200
	2	512	128	256	200	1600
	3	128	16	256	1600	800
	4	512	128	512	1600	800
	Average	352	102	320	900	850

Figure 10. Immune response of rabbits to Trichinella spiralis as measured by the complement fixation test. Ranges are shown by the vertical lines.


512
256
128
64
32
16
8
Whole Serum
IgM
IgG
Complement Fixation Titer
0
7
14
21
28
35
42
49
56
Days post gavage

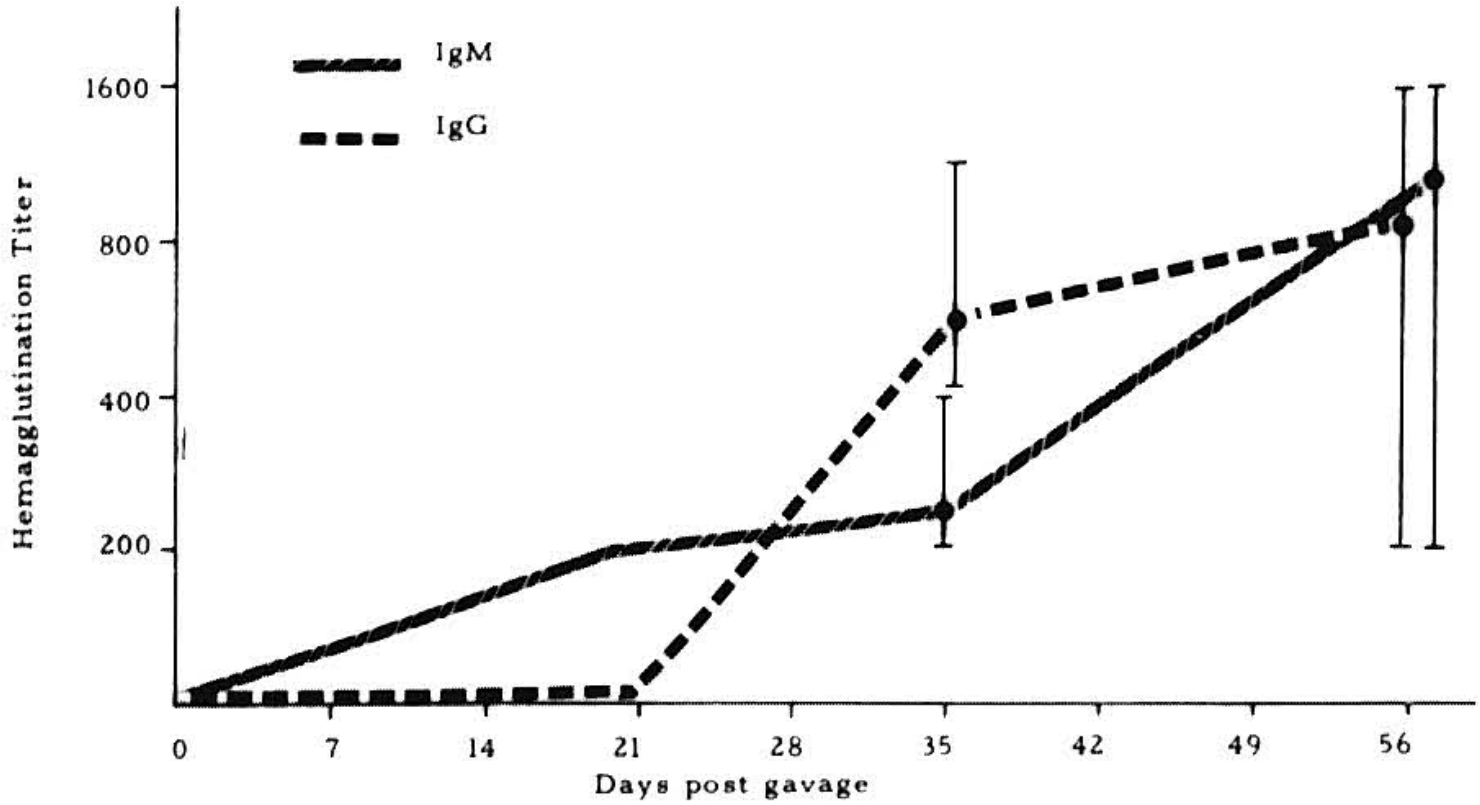


Figure 11. Immune response of rabbits to Trichinella spiralis as measured by the hemagglutination test. Ranges are shown by the vertical lines.

after the second gavage had a IgM titer of 250 and IgG titer of 600. Twenty-one days after the third gavage, the IgM and IgG titers were approximately the same (IgM 900, IgG 850).

Studies in Mice of Larvae Incubated in Serum and Serum Fractions

Samples were prepared as follows: 0.3 ml of whole serum containing 60 µg/ml of protein, 0.3 ml of IgM containing 1.0 µg/ml of protein and 0.3 ml of IgG containing 9 µg/ml of protein (Crandall and Moore, 1968). Each was inactivated at 56°C for 30 minutes. These quantities of protein of IgM and IgG were used, because they are the equivalents of the immunoglobulins contained in the whole serum. To each of these inactivated samples, 500 live T. spiralis larvae were added and incubated for 18 hours at 37°C in a water bath. After incubation, the larvae were washed with physiological saline to remove any unreacted antibodies. The treated larvae were pooled according to groups of immune and normal whole serum, immune and normal IgM and immune and normal IgG, and resuspended in saline to yield 100 larvae /0.1 ml volume. This determined number of larvae and volume of suspending medium was the

gavage dose per mouse. These larvae were still motile as determined by microscopic examination.

One hundred and twenty, 13 to 20 grams mice were randomly divided into six groups, three groups were to receive larvae incubated in normal serum and fractions, and the other three to receive larvae incubated in immune serum and fractions. Each mouse was gavaged with 100 preincubated larvae. The mice were identified by ear punch. Twenty mice received larvae incubated for 18 hours in one of the following:

Normal whole serum

Normal IgM

Normal IgG

Immune whole serum

Immune IgM

Immune IgG

Ten mice from each of the six groups were sacrificed 72 hours post-gavage for the recovery of adult worms. This was accomplished by removing the small intestines, slitting them longitudinally and digesting them in 0.1% NaOH overnight at 4°C.

There was a 7-fold decrease in the average number of adult worms recovered from the mice which received larvae

that were incubated in immune whole serum as compared to those that received larvae preincubated in normal whole serum (12.7 vs 1.7, $p < 0.01$, Table III). When the mean number of worms recovered from mice which received larvae incubated in immune IgM was compared to mice that received larvae incubated in normal IgM, there was a 38-fold decrease (3.8 vs 0.1, $p < 0.01$, Table IV). Eight of the nine mice gavaged with larvae incubated in immune IgM, had no worms in the intestinal tract. There was no difference in the number of adult worms recovered from immune IgG and normal IgG groups (7.8 vs 7.6, $p > 0.1$, Table V).

In those animals surviving the 28 days before sacrificing for larva counts, weight gain was observed in all the animals (Tables VI, VII, and VIII). The animals that received larvae incubated with immune IgM on the average gained the most weight. However, there was no difference between the average weight gain in grams among the animals that received the larvae incubated in immune whole serum as compared to those that received larvae incubated in normal whole serum (Tables VI, VII, VIII).

Table III. Number of adult worms recovered from intestine of mice 72 hours post-gavage. The larvae had been preincubated in normal and immune whole serum.

NORMAL WHOLE SERUM					IMMUNE WHOLE SERUM				
Mouse No.	Initial weight (gms)	Gavage weight (gms)	72 hr post-gavage weight (gms)	No. of adult worms	Mouse No.	Initial weight (gms)	Gavage weight (gms)	72 hr post-gavage weight (gms)	No. of adult worms
1	16	18	16	13	1	16	10	died	--
2	13	16	13	11	2	16	10	died	--
3	16	16	14	17	3	14	12	13	2
4	15	17	17	12	4	15	13	died	--
5	16	16	16	16	5	13	15	18	2
6	16	14	18	8	6	15	14	20	2
7	14	21	20	14	7	14	14	13	6
8	17	19	18	11	8	14	18	16	0
9	16	18	died	--	9	19	20	14	0
10	14	16	18	12	10	16	12	18	0
Average	15.3	17.1	16.6	12.7		15.2	13.8	16.0	1.7
S. D.	1.30	1.99	2.18	2.70		1.69	3.22	2.76	2.14
Number of negative animals				0/9					3/7

p of adult worms < 0.01

Table IV. Number of adult worms recovered from intestine of mice 72 hours post-gavage. The larvae had been preincubated in normal and immune IgM.

NORMAL IgM					IMMUNE IgM				
Mouse No.	Initial weight (gms)	Gavage weight (gms)	72 hr post-gavage weight (gms)	No. of adult worms	Mouse No.	Initial weight (gms)	Gavage weight (gms)	72 hr post-gavage weight (gms)	No. of adult worms
1	16	19	20	0	1	16	20	20	0
2	16	17	16	5	2	14	18	died	--
3	17	16	16	4	3	14	16	14	0
4	14	19	21	4	4	16	13	15	0
5	14	16	20	3	5	16	16	18	0
6	15	18	20	4	6	16	16	18	0
7	16	19	18	3	7	14	15	17	0
8	15	18	26	6	8	16	18	18	1
9	16	20	died	--	9	12	18	19	0
10	15	18	16	5	10	18	20	20	0
Average	14.9	17.4	19.2	3.8		15.2	17.3	17.3	0.1
S. D.	1.14	1.70	3.24	1.72		1.70	2.20	2.14	0.33
Number of negative animals				1/9					8/10

p of adult worms < 0.01

Table V. Number of adult worms recovered from intestine of mice 72 hours post-gavage. The larvae had been preincubated in normal and immune IgG.

	NORMAL IgG					IMMUNE IgG			
Mouse No.	Initial weight (gms)	Gavage weight (gms)	72 hr post-gavage weight (gms)	No. of adult worms	Mouse No.	Initial weight (gms)	Gavage weight (gms)	72 hr post-gavage weight (gms)	No. of adult worms
1	14	14	lost	--	1	15	15	19	2
2	15	18	19	10	2	14	13	15	4
3	18	19	16	9	3	14	18	20	6
4	14	15	10	7	4	16	16	17	10
5	16	17	11	6	5	16	18	21	12
6	14	12	12	0	6	16	10	lost	--
7	16	12	11	6	7	16	14	died	--
8	10	11	died	--	8	15	18	21	9
9	13	16	died	--	9	13	12	20	11
10	13	10	14	15	10	15	24	24	8
Average	14.3	14.4	13.3	7.6		15.0	15.8	19.6	7.8
S. D.	2.16	3.13	1.28	4.58		1.05	3.96	2.70	3.49
Number of negative animals				1/7					0/8

p of adult worms > 0.1 N. S.

Table VI. Number of larvae recovered from musculature of mice 28 days post-gavage. The larvae had been preincubated in normal and immune whole serum.

NORMAL WHOLE SERUM					IMMUNE WHOLE SERUM				
Mouse No.	Initial weight (gms)	Gavage weight (gms)	28 days post-gavage weight (gms)	No. of larvae	Mouse No.	Initial weight (gms)	Gavage weight (gms)	28 days post-gavage weight (gms)	No. of larvae
1	16	16	20	9,100	1	16	18	22	8,100
2	15	15	18	3,350	2	16	18	25	2,800
3	16	18	21	1,300	3	13	16	died	----
4	16	21	24	4,342	4	18	20	died	----
5	13	17	died	----	5	16	18	23	1,700
6	16	21	25	17,800	6	16	18	21	2,200
7	13	16	lost	----	7	12	16	25	4,700
8	14	18	died	----	8	14	18	died	----
9	17	16	21	15,000	9	17	20	23	2,380
10	14	15	30	4,300	10	12	16	lost	----
Average	15.0	17.3	22.7	7,882		15.0	17.8	23.1	3,646
S. D.	1.41	2.21	4.01	6,323		2.10	1.47	1.60	2,414
Number of negative animals				0/7					0/6

p of larvae > 0.1 N. S.

Table VII. Number of larvae recovered from musculature of mice 28 days post-gavage. The larvae had been preincubated in normal and immune IgM.

NORMAL IgM					IMMUNE IgM				
Mouse No.	Initial weight (gms)	Gavage weight (gms)	28 days post-gavage weight (gms)	No. of larvae	Mouse No.	Initial weight (gms)	Gavage weight (gms)	28 days post-gavage weight (gms)	No. of larvae
1	16	17	25	6,000	1	13	20	28	0
2	16	16	24	2,600	2	16	17	27	0
3	13	20	26	2,500	3	16	14	25	0
4	16	17	23	6,400	4	14	20	30	0
5	14	14	23	1,100	5	17	19	28	0
6	14	16	26	900	6	16	15	27	40
7	14	20	22	800	7	16	20	32	150
8	16	17	lost	----	8	13	14	31	0
9	14	14	lost	----	9	18	20	27	0
10	13	18	18	500	10	13	18	27	0
Average	14.6	16.9	23.3	2,600		15.2	17.7	28.2	19.0
S. D.	1.26	2.07	2.64	2,354		1.81	2.54	2.13	47.71
Number of negative animals				0/8					8/10

p of larvae < 0.01

Table VIII. Number of larvae recovered from musculature of mice 28 days post-gavage. The larvae had been preincubated in normal and immune IgG.

NORMAL IgG					IMMUNE IgG				
Mouse No.	Initial weight (gms)	Gavage weight (gms)	28 days post-gavage weight (gms)	No. of larvae	Mouse No.	Initial weight (gms)	Gavage weight (gms)	28 days post-gavage weight (gms)	No. of larvae
1	16	24	29	18,000	1	16	19	25	5,500
2	14	16	32	4,000	2	13	18	25	20,930
3	19	20	23	10,000	3	15	19	21	17,200
4	14	10	died	----	4	13	13	died	----
5	16	15	27	4,700	5	18	12	19	2,000
6	16	18	23	4,200	6	14	14	died	----
7	18	20	died	----	7	13	13	21	7,800
8	14	17	23	9,180	8	15	16	27	1,000
9	16	19	29	11,600	9	20	20	19	7,200
10	15	19	25	2,520	10	13	17	died	----
Average	15.7	17.8	26.3	8,225		15.0	16.1	22.4	8,804
S. D.	1.76	3.70	3.42	5,474		2.40	2.93	3.18	7,519
Number of negative animals				0/8					0/7

p of larvae > 0.1 N. S.

The larvae recovered from the musculature of the various mice at 28 days after infection revealed a similar pattern in the number of adult worms recovered at 72 hours post-gavage. An average of 3,646 larvae was recovered from mice that received the larvae treated with immune whole serum versus an average of 7,882 larvae from mice which were given larvae treated with normal whole serum (Table VI). Although there is a 46% decrease in the average number of larvae recovered in the immune group, the figures are not statistically significant ($p > 0.1$). This is due to the number of larva-recovery variance among the mice. The difference in the average number of larvae recovered from mice which received larvae treated in immune IgM and normal IgM was statistically highly significant ($p < 0.01$), and the average numbers being 2,600 versus 19 respectively (Table VII). Eight of the ten mice gavaged with immune IgM treated Trichinella yielded no larvae. The same findings were recorded for the adult worm recoveries from the mice that received the larvae preincubated in immune IgM. There was no significant difference between the number of larvae obtained in the normal IgG group and immune IgG group (8,225 vs 8,804, $p > 0.1$, Table VIII).

DISCUSSION

In the present study, it was found by the use of the complement fixation test that the two classes of anti-Trichinella antibodies coexist, with the IgM being lower than IgG but parallel to each other and to the total antibody titer as indicated in the whole serum. As measured by the passive hemagglutination test, the IgM were the only antibodies detected in the 21-day sample, while IgG antibodies appeared first in the 35th day sample after the first gavage (14 days after the second gavage). The level of the IgM antibodies in these rabbits did not decline throughout the course of the experiment. Crandall and Moore (1968), by using the passive hemagglutination test, with Melcher's trichina antigen, also showed that the first detectable antibody following a single infection with T. spiralis larvae was associated with IgM. At three weeks after the infection, IgG antibodies were not present. However, by the fourth to sixth weeks, IgG antibodies began to appear and persisted at relatively high titer for at least four months after the larvae were administered. Both IgM and IgG antibodies were present in rabbits after multiple challenges. These authors concluded that they could not definitely establish an early rise of IgM anti-Trichinella antibody, followed by IgG antibodies in rabbits, because in the hemagglutination test, it was shown that antibodies are more efficient than IgG antibodies. Onoue et al (1965) reported that the IgM

was about 180 times more efficient in agglutination on a molar basis than that of IgG. This might be due to the presence of six antigen binding sites on the IgM as compared to the two on the IgG. Freeman and Stavitsky (1965) postulated that the apparent sequence of production of IgM and IgG antibodies may be only a reflection of the greater efficiency of the macroglobulin antibody in the hemagglutination test.

On the basis of my findings reported here, it was shown that both the immune IgM and the total immune globulins of the whole serum had adverse in vitro effects upon the trichina larvae fed to the mice, especially on the development to adult worms, and resulted in the majority of the mice from being infected. There was no difference in effect observed in the larvae treated with immune IgG or normal IgG. A statistical analysis of the comparison between the treatment of larvae with (1) immune IgM and immune IgG, (2) immune whole serum and immune IgM, and (3) immune whole serum and immune IgG showed that in the first comparison (immune IgM versus immune IgG) there was a significant difference in the number of adult worms recovered (0.1 vs 7.8, $p < 0.01$), and in the number of secondary larvae recovered (19 vs 8,804). In the second comparison, the immune IgM was statistically more effective than immune whole serum, both in the average number of adult worms and daughter larvae recovered (0.1 vs 1.7 and 19 vs 3,646, $p < 0.01$). There was a significant difference in the third

comparison in the average number of adult worms recovered after treatment with immune whole serum and immune IgG (1.7 vs 7.8, $p < 0.01$), but there was no significant difference in the number of larvae recovered (3,646 vs 8,804, $p > 0.1$). This latter insignificant difference is undoubtedly due to the large variance in the number of larvae recovered (Table IX). The above data are interpreted to show that it is the IgM fraction of the immune serum that has the most adverse effects on the infectivity of the larvae of T. spiralis.

The complement fixation test and the in vitro precipitin reaction of live T. spiralis larvae with immune IgM showed a lower reaction than the immune IgG. This means that neither the complement fixing ability nor the amount of precipitate formed in the precipitin reaction indicates the presence of protective antibodies in the serum. Soulsby (1962) and Tanner (1968) concluded that despite the extensive amount of work which has been done and is still in progress on various serological manifestations in nematode infections, no convincing evidence exists to show that antibodies detected by numerous serological tests play any functional part in immunity (protection). The antibody detected by the serological tests serve only as a guide to the antibody response to parasitic infections.

The mechanism of action of the immune IgM upon the vitality of T. spiralis larvae can be explained on the basis that the immune

Table IX. Summary of adult worm and secondary larva recoveries, giving comparison and level of significance (in parenthesis) of immune whole serum and fractions.

	Average No. adult worms		Average No. larvae	
Immune IgM	0.1		19	
(1)		($p < 0.01$)		($p < 0.01$)
Immune IgG	7.8		8,804	
Immune whole serum	1.7		3,646	
(2)		($p < 0.01$)		($p < 0.01$)
Immune IgM	0.1		19	
Immune whole serum	1.7		3,646	
(3)		($p < 0.01$)		($p > 0.1$)
Immune IgG	7.8		8,804	

fraction may alter the basic metabolism of the parasite, food intake, respiration, or may physically clog the various openings of the larvae by the formed precipitate. Oliver-Gonzalez (1940) interpreted the clogging to mean that the precipitates at the mouth of the larvae were too large for the larvae to release. This resulted in immobilization and death of the larvae. Larsh (1967) concluded that the interference with metabolic activity resulted in stunting of the worms, and might even inhibit the normal reproductive potential of the worms during infection. However, Komandarev (1963) indicated to the contrary that the reproductive capacity and the number of muscle trichina larvae were not affected by the immune gamma globulin.

In Trichinella infection, the immunity is transferred from host mother to young mainly in the milk of the infected mother (Culbertson, 1943). The young born of a normal, uninfected mother rat can promptly become immune if they are permitted to feed from the immune mother, while conversely, the young born of an immune mother rat are susceptible to T. spiralis infection when they are fed with the milk of a normal mother rat. It is evident here that immunity in T. spiralis is not transplacental, and therefore it is not associated with IgG immunoglobulin, since Kunkel (1960) showed that only IgG passes the placental barrier.

SUMMARY

The complement fixation and hemagglutination tests were used to determine the immune response of rabbits to Trichinella spiralis. It was found by the complement fixation test that the antibodies manifested as IgM and IgG were present concurrently and persisted over the period of 56 days, during which time three gavages with live T. spiralis larvae were given.

The IgM and IgG immunoglobulins were fractionated by Sephadex G-200 gel filtration. The peaks from gel filtration were identified by immunoelectrophoresis showing the first peak to be IgM and the second IgG.

Mice were given 100 live muscle-free Trichinella spiralis larvae by gavage. The larvae had been preincubated in either (1) normal whole serum, or immune whole serum; (2) normal IgM or immune IgM; (3) normal IgG or immune IgG.

The precipitin reaction using serum or immunoglobulins on the larvae in vitro showed that there was a 4+ precipitate in the medium and on the larvae incubated in immune serum and immune IgG. There was only a 2+ precipitate in the medium of immune IgM. No precipitate was observed when larvae were incubated in normal serum, normal IgM or normal IgG.

When the pretreated larvae were fed to mice, the following

data on infectivity and development were obtained:

1. There was a significantly smaller number of adult worms recovered when the mice were given larvae incubated in immune whole serum and immune IgM, than when the larvae were incubated in normal whole serum and normal IgM. No difference in the number of adult worms recovered was observed when the larvae were pretreated with immune IgG and normal IgG.

2. The number of secondary larvae recovered after immune IgM treatment was significantly smaller than was recovered after the normal IgM treatment. In comparison the average number of larvae recovered from the immune whole serum group was half as many as from the normal whole serum. Statistically, there was no difference. There was no difference between the two IgG groups (normal and immune).

3. There were significant reductions in the number of both adult worms and secondary larvae recovered from mice which received larvae preincubated in immune IgM as compared to immune IgG as well as in immune IgM versus immune whole serum. The number of adult worms recovered after immune whole serum treatment was significantly less than those after the immune IgG, but no difference was found in the number of secondary larvae recovered.

On the basis of this study, the fraction of immune rabbit serum

which had the greatest physiologically adverse effects on the infective stage of T. spiralis larvae, and impeded their infectivity and development in mice to adult worms, was found in the IgM immunoglobulin. This was accomplished by first incubating the live larvae in the IgM at 37°C for 18 hours and then feeding them to the mice.

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