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The Immunological Response of Mice Infected with *Trichinella spiralis*

BIOLOGICAL AND PHYSICO-CHEMICAL DISTINCTION OF TWO HOMOCYTOTROPIC ANTIBODIES

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Summary. (1) Infection of mice with *Trichinella spiralis* induced the appearance in serum of two homocytotropic antibodies that could be distinguished by their biological and chromatographic behaviour.

(2) Biologically, the antibodies could be distinguished by their ability to persist in homologous skin after passive transfer. One antibody was able to induce PCA only when a short latent period was used, whereas the other was able to induce PCA even after 72 hours.

(3) They could also be separated when antiserum was passed through a DEAE-cellulose column. Antibody present in the first eluates was able to induce PCA only if a short latent period was used whereas antibody present in the subsequent eluates was able to induce PCA 72 hours after sensitization.

(4) Both antibodies appeared in the circulation 5 weeks after infection and reached their highest levels around the 9th week. Later, the 72-hour PCA antibody disappeared from the serum in some animals, whereas the 4-hour PCA antibody remained.

(5) Re-infection resulted in an increase in the levels of both antibodies.

(6) In animals subjected to repeated reinfections the reagin-like antibody either decreased or disappeared from the serum. On the other hand, the 4-hour PCA antibody increased.

(7) Immunization with 'dead' *T. spiralis* antigen led to the appearance of both antibodies in the serum. A second dose of antigen resulted in increases in the levels of both antibodies, but further injections resulted in a high level of 4-hour PCA antibody and in the disappearance of the reagin-like antibody.

INTRODUCTION

The antibody formed even to a single antigen may consist of a population of molecules with varying physico-chemical properties and varying biological activity. Recent studies on the biological behaviour of these different antibody molecules led to the finding that some of the antibodies produced by a given species are not able to mediate anaphylaxis in the same species although they do so in other species (Ovary, Benacerraf and Bloch, 1963; Nussenzweig, Merryman and Benacerraf, 1964; Barth and Fahey, 1965). Conversely, other immunoglobulins were shown to be able to sensitize only the same species

that produced them or a closely related species. These latter antibodies were named homocytotropic while the former were named heterocytotropic (Becker and Austen, 1968). Some homocytotropic antibodies are characterized by their sensitivity to heat and to alkylation and reduction as well as by their remarkable property of remaining in the sensitized skin for many days. It is this property of skin persistence which provides the most specific means of characterization of this type of homocytotropic antibody which will be named throughout this paper 'reagin-like antibody'. This type of antibody is usually present at a high level in the serum of animals infected with helminthic parasites.

The recent observation that after a single antigenic stimulation mice can produce two distinct homocytotropic antibodies, one able to induce passive cutaneous anaphylactic (PCA) reactions only within a few hours after sensitization and another sensitizing for PCA even after a 72-hour latent period (Mota, 1967), prompted us to investigate the production of similar antibodies by mice infected with *Trichinella spiralis*.

MATERIALS AND METHODS

Animals

ICR, Bagg and hairless mice bred at Walter Reed Army Institute of Research, weighing 18–25 g, were used throughout. The principles of animal care as promulgated by the National Society for Medical Research were observed.

Parasitic infection

Each mouse was fed 100 washed *T. spiralis* larvae either in the primary or later infections. The larvae were obtained from young adult rats approximately 6 weeks after they were fed 3000–4000 washed *T. spiralis* larvae. The rats were killed and the larvae were separated from the muscles by artificial digestion in a pepsin–hydrochloric acid mixture.

Preparation of antigens

Extract of T. spiralis *larvae.* A lipid-free somatic extract of *T. spiralis* larvae was prepared at 4° in buffered saline (Chaffee, Bauman and Shapilo, 1954). Lyophilized aliquots of the finished product were sealed in vials and stored at 4°. Solutions were made when needed by dissolving the dry extract in 0·85 per cent NaCl.

Metabolic antigen. Larvae were collected from the ground meat of infected rats after digestion in pepsin–HCl mixture for 90–120 minutes. They were washed at least six times by sedimentation in distilled water, and then counted by the dilution method and placed in a 1 : 10,000 merthiolate–physiological saline solution for 10 minutes. After two more washings in sterile physiological saline solution, 500,000 larvae were transferred into each of several 100-ml Erlenmeyer flasks containing 15 ml of medium 19 (Microbiological Associates, Inc.) with 20 per cent foetal bovine serum, 15,000 units of penicillin and 15 mg of streptomycin. A drop of the culture in each flask was examined under the microscope, and if dead larvae were found the batch was discarded. If no dead larvae were present the mixture was shaken gently and incubated at 37° for 72 hours. At the end of this time practically all of the larvae were motile. The few which were immobile showed no visible signs of internal degeneration. The larvae were separated from the preparation by high speed centrifugation and the supernatant fluid was stored at −20° until used as antigen.

Somatic antigen. Larvae were collected from infected rats as above. They were washed by sedimentation in five changes of distilled water. Without further delay, they were

placed in a Ten Broeck tissue grinder (Arthur H. Thomas, Philadelphia) in an ice bath and homogenized for 20 minutes and used immediately as antigen.

Antiserum. Blood was obtained either at the end of the experiment by cutting the brachial plexus under ether anesthesia and collecting the extravasated blood with a Pasteur pipette, or during the course of immunization by puncture of the ophthalmic venous plexus. The blood was allowed to clot in an ice-bath and the serum separated by centrifugation in a refrigerated centrifuge. Sera were kept at $-20°$ until used.

Passive cutaneous anaphylaxis (PCA)

Ovary's method for PCA (Ovary, 1958) was used for detecting and estimating antibody with the exception that a latent period of 72 hours was also used (Mota, 1963a). Two or three intradermal injections of 0·05 ml antiserum or antiserum dilution were made on each side of the mouse's dorsal skin with a sharp hypodermic needle, and PCA reactions were elicited after a latent period of either 4 or 72 hours. The animals were injected intravenously with 0·5 ml of a 0·25 per cent solution of Pontamine Sky Blue in saline containing 0·5 mg antigen. Twenty to 30 minutes after antigen injection the animals were killed with ether, the skin was inverted and the lesion diameter was measured on the inner surface of the skin with a transparent ruler. Antibody was estimated by determining the highest dilution of antiserum which induced a PCA reaction. PCA titres were stated as the reciprocal of the highest serum dilution giving a skin reaction. A minimum of six mice was used for each determination. Hairless mice were most frequently used for PCA reactions because they did not need to be shaved and because they provided more consistent PCA reactions than either Bagg or ICR strains of mice. However, due to difficulty in obtaining hairless mice in sufficient numbers, Bagg mice were occasionally used.

Treatment with mercaptoethanol

Samples of serum (0·5 ml) were dialysed against 250 ml of 0·1 M 2-mercaptoethanol for 3 hours at room temperature and then dialysed against 500 ml 0·02 M iodoacetamide for 4 hours. Before testing, samples were dialysed against several changes of phosphate-buffered saline for 18–24 hours at 4°. Control samples were either dialysed against phosphate-buffered saline in place of mercaptoethanol and then treated with iodoacetamide as described above, or dialysed only against phosphate-buffered saline.

Treatment with heat

To determine the effect of heating on the ability of anti-*T. spiralis* mouse serum to induce PCA, the antiserum was placed for 60 minutes in a water bath at 56°.

Chromatography on DEAE-cellulose

Chromatography on DEAE-cellulose was performed by applying 3 ml antiserum to columns measuring 2·5 cm in diameter and 30 cm in length packed under 5 lb of pressure. Gradient elution was performed by gradually combining three phosphate buffer solutions having the same pH of 8·0 but differing in their molarity by addition of NaCl as follows: 0·01 M, 0·1 M and 0·15 M. The mixture of these solutions was made by using the variable gradient device for chromatography as described by Peterson and Sober (1959). Three-millilitre samples were collected at the rate of about 0·7 ml/min. The protein concentration of the eluates was determined by spectrophotometry at 2800 Å; protein peaks were combined

and their volume was reduced to the original serum volume by positive pressure filtration. The concentrated samples were then tested for their ability to induce PCA either 4 or 72 hours after sensitization.

RESULTS

INDUCTION OF ANTIBODY PRODUCTION BY INFECTION WITH *T. spiralis*

The appearance and persistence of antibodies after a single infection with *T. spiralis* were studied in two groups of animals infected in the same way, approximately 2 weeks apart. The animals of each group were bled weekly from the 4th to the 16th weeks after infection. The individual sera of each weekly bleeding were pooled and tested for their ability to induce PCA reactions after a latent period of 4–72 hours. In both groups, 4- and 72-hour PCA antibody activity appeared simultaneously in the circulation 5 weeks after infection and each attained its maximum around the 9th week. From then on there was a striking difference between the two groups. In one group (Fig. 1) the antibody responsible

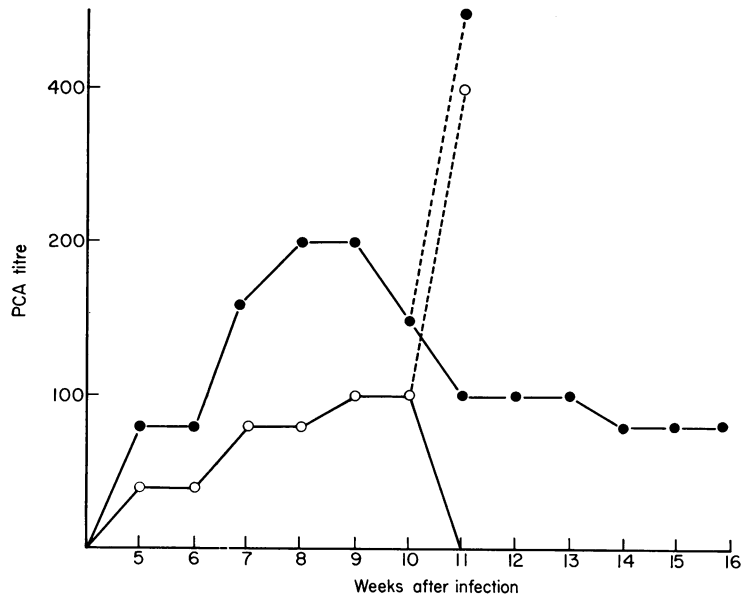

FIG. 1. Time course of 4-hour (●) and 72-hour (○) PCA activity of mouse anti-*T. spiralis* serum obtained at different times after infection. The broken lines show antibody level after re-infection,

for the 72-hour PCA reaction disappeared from the serum after the 10th week whereas the 4-hour PCA antibody persisted throughout the duration of the experiment. In the other group (not shown here) both antibodies remained in circulation 16 weeks after infection when the animals were killed. At that time the reagin-like antibody content was very low (PCA titre of 10 after 72 hours).

Effect of a second infection

Ten weeks after the primary infection of the first group of mice they were divided into two groups, one of which received a second infection. The other group was not re-infected

but was kept as a control to test the antibody level in the absence of re-infection. A week later both groups were bled and the pooled sera of each group were tested for PCA activity after 4 and 72 hours. Both of these activities rose sharply a week after the secondary infection (Fig. 1, broken lines).

Effect of repeated infections

In order to observe the effect of multiple infections, a group of mice was infected with *T. spiralis* and bled from the ophthalmic plexus for antibody determinations 9 weeks later. The animals were then re-infected six times, at weekly intervals. Antiserum was collected 6 days after the third re-infection and 10 days after the sixth re-infection. Antisera obtained after the primary infection and after the third re-infection were pooled while antisera

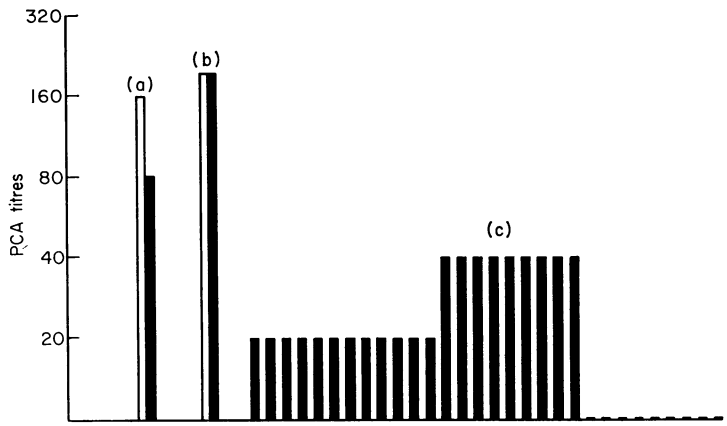

FIG. 2. Antibody level of sera of *T. spiralis* infected mice: (a) Four- (open columns) and 72- (solid columns) hour PCA titre of pooled sera obtained 9 weeks after a primary infection; (b) 4- and 72-hour PCA titre of pooled sera obtained 1 week after the last of three additional infections; and (c) 72-hour PCA titre of individual sera obtained 10 days after the sixth and last additional infection. Each vertical column represents one serum.

obtained after the last re-infection were tested individually. The results of this experiment, summarized in Fig. 2, show that although the level of the reagin-like antibody increased after three re-infections, it was very much reduced after the sixth re-infection and had disappeared from the sera of some of the animals. On the other hand, the 4-hour PCA antibody was still detectable at high titres even after the sixth re-infection (six of these sera had a 4-hour PCA titre of 200 and eight others had a titre of 100).

Induction of antibody production by 'dead' T. spiralis *antigen*

Although it was realized that it is very difficult if not impossible to duplicate by artificial immunization the same type of antigenic stimulation provided by a living infection, experiments were performed to find out whether immunization of mice with 'dead' *T. spiralis* antigens would induce an immunological response similar to that induced by a living parasite infection. A group of mice received a single subcutaneous dose of 100 μg *T. spiralis* larval antigen adsorbed onto aluminum hydroxide. Ten days later the animals were bled from the ophthalmic plexus and their sera were pooled and tested for PCA 4 and 72

TABLE 1

PRODUCTION OF ANTIBODIES TO *T. spiralis* ANTIGEN BY MICE AFTER SINGLE OR MULTIPLE INJECTIONS OF 'DEAD' ANTIGEN

Type of antigen	No. of injections	Amount of antigen injected	PCA titre	
			4-hour	72-hour
Larval extract	1	100 μg	50	20
	3	50 μg	100	100
	6	50 μg	200	0
Fresh washed larvae	1	10^3 larvae	20	5
Metabolic antigens	1	$1{\cdot}5 \times 10^6$ larvae	20	10

Antibody content of antisera determined by PCA reactions induced 4 or 72 hours after sensitization.

hours after sensitization. The results of this experiment showed that immunization with an extract of *T. spiralis* larvae was able to induce the production of antibody activities with the same PCA characteristics as those present in the serum of mice infected with *T. spiralis* (Table 1).

Effect of repeated doses of antigen

The same animals used in the previous experiments subsequently received six intradermal injections at weekly intervals of 50 μg *T. spiralis* antigen in 0·1 ml of saline. Seven days after the third injection and 10 days after the last injection, they were bled and their sera pooled. The pools were then tested for their ability to induce PCA 4 and 72 hours after sensitization. The content of the 72-hour PCA antibody increased after two additional injections of antigen and then disappeared after three subsequent injections (Table 1). However, the same treatment resulted in a steady increase in the 4-hour PCA antibody.

IMMUNIZATION WITH FRESH LARVAE SUSPENSION AND METABOLIC ANTIGENS

Since drying and lyophilization of larvae can produce denaturation of larval antigens that may be important for the induction of reagin-like antibodies, a group of mice was injected with 0·5 ml of a freshly-prepared *T. spiralis* larvae suspension (1000 larvae/ml) plus aluminum hydroxide.

Metabolic antigens which are products of excretion and secretion of the worms are present in relatively low concentration in an extract of larvae. Since these antigens are constantly produced in the infected organism they may represent an important antigenic stimulus missing in the larval extract. Accordingly, a group of mice was also injected with 0·25 ml of the metabolic antigen preparation plus aluminum hydroxide. The results of these experiments (Table 1) showed that neither the fresh larvae suspension nor the metabolic antigens preparation were more effective in inducing the production of reagin-like antibodies than the dried and lyophilized extract of larvae.

Effect of treatment with 2-mercaptoethanol

As shown in Table 2 treatment of mouse anti-*T. spiralis* sera with 2-mercaptoethanol followed by alkylation with iodoacetamide resulted in a considerable decrease in the ability of antisera to induce PCA after 4 hours and in the total loss of their ability to induce PCA after 72 hours.

TABLE 2

THE EFFECT OF REDUCTION AND ALKYLATION ON PCA REACTIVITY OF SERA FROM MICE INFECTED WITH *T. spiralis*

Treatment	Average PCA titres*			
	Untreated		Treated	
	4-hour	72-hour	4-hour	72-hour
2-Mercaptoethanol	80	50	80	50
Iodoacetamide	80	50	80	50
Dialysis only	80	50	80	50
Mercaptoethanol and iodoacetamide	80	50	10	0

* Reciprocal of highest antiserum dilution giving a skin reaction.

TABLE 3

THE EFFECT OF HEAT ON PCA REACTIVITY OF SERA FROM MICE INFECTED WITH *T. spiralis*

Antiserum	PCA titres*			
	Unheated		Heated (56° 1 hour)	
	4-hour	72-hour	4-hour	72-hour
TW	200	100	200	0
SP	400	50	400	0
TI	80	40	80	0
TB	400	400	400	0

* Reciprocal of highest antiserum dilution giving a skin reaction.

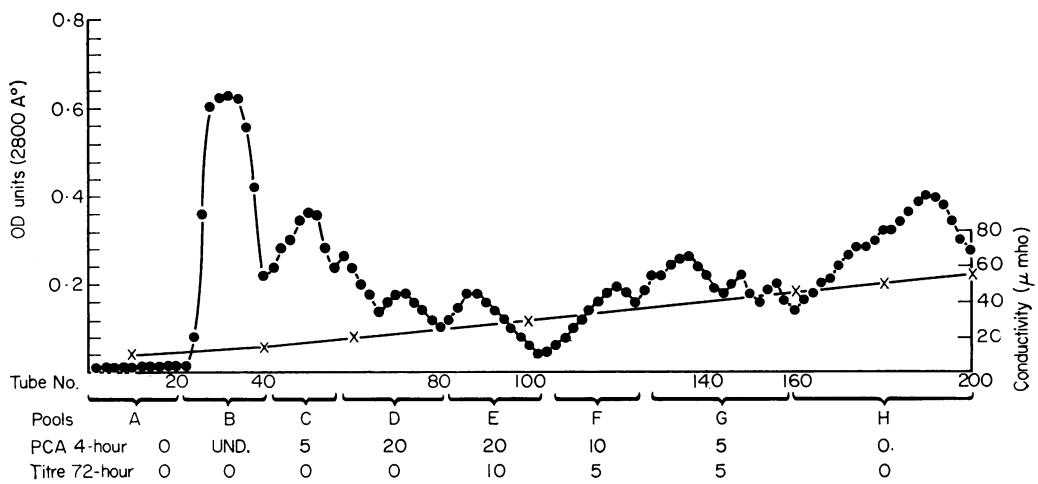

FIG. 3. Activity of pooled eluates obtained by DEAE-cellulose chromatography of mouse anti-*T. spiralis* serum. Each pool was tested for its ability to induce PCA 4 and 72 hours after sensitization. ●, Protein concentration; ×, conductivity.

Effect of heating

The effect of heating at 56° for 60 minutes on the reactivity of different mouse anti-*T. spiralis* sera is shown in Table 3. Although the ability of the antisera to induce PCA after 4 hours was not changed, their ability to induce the same reaction after 72 hours was completely lost.

Chromatographic separation of the 4- and 72-hour PCA activity

Chromatography of mouse anti-*T. spiralis* serum on a DEAE-cellulose column resulted in some eluates able to induce PCA only after 4 hours and others able to induce PCA after 4 and 72 hours. Fig. 3 shows the distribution of PCA activity in the several eluates obtained using mouse anti-*T. spiralis* pool T collected 6 weeks after a single infection (PCA titre after 4 hours: 80; after 72 hours: 40).

DISCUSSION

Reagin-like antibodies are known to occur under natural conditions in man and lower animals and their artificial production has been obtained recently in laboratory animals such as rats (Mota, 1963a, b; Binaghi and Benacerraf, 1964; Binaghi, Benacerraf, Bloch and Kourilsky, 1964), rabbits (Zvaifler and Becker, 1966) and mice (Mota and Peixoto, 1966; Mota, 1967; McCamish, 1967). These antibodies have been designated by a variety of terms: reagin-like, mast cell sensitizing, anaphylactic, passive cutaneous anaphylactic and homocytotropic antibodies. For reasons still undetermined animals infected with helminthic parasites produce large quantities of these antibodies whose role in host resistance is not yet precisely known. The presence of relatively high levels of a reagin-like antibody has been detected in the serum of rats infected with *Nippostrongylus brasiliensis* (Ogilvie, 1964); of humans, chimpanzees and rhesus monkeys infected with *Schistosoma mansoni* (Sadun, von Lichtenberg, Hickman, Bruce, Smith and Schoenbechler, 1966); of rabbits infected with *S. mansoni* (Zvaifler, Sadun, Becker and Schoenbechler, 1967) and of rabbits infected with *Dirofilaria uniformis* (Sadun, Duxbury, Gore and Stechschulte, 1967). Previous indirect evidence for the existence of a 'mast cell sensitizing' antibody in mice infected with *T. spiralis* was provided by Briggs (1963) who showed that mast cells of mice infected with *T. spiralis* were disrupted when contacting specific antigen. Our results show that mice infected with *T. spiralis* produce at least two homocytotropic antibodies, one able to induce PCA reactions only within a few hours after sensitization and another sensitizing for PCA even after a 72-hour latent period. Like the early antibody induced by injecting mice with ovalbumin (Mota, 1968), the 72-hour PCA antibody obtained from *T. spiralis* infected mice is completely destroyed by heating and by treatment with mercaptoethanol. On the other hand, PCA reactions induced 4 hours after sensitization were not changed by heat and were only partially reduced by treatment with mercaptoethanol. The antibodies responsible for these activities not only differ in their biological properties but also have different physicochemical properties which allow their separation by chromatography on DEAE-cellulose. As can be seen in Fig. 3, eluates B, C and D were able to induce PCA after 4 hours but not after 72 hours. Actually they were not able to induce PCA even when challenged as early as 24 hours after sensitization. Thus, the antibody eluted with the first fractions is not able to remain in the skin for more than a few hours. On the other hand, eluates E, F and G were able to induce PCA after 4 hours and after 72 hours. PCA activity in these eluates may be due to only one antibody able to

induce PCA after 4 hours as well as after 72 hours or may be due to two antibodies, one responsible for the 4-hour PCA and another responsible for the 72-hour PCA. Thus, it is possible that these different eluates still represent a heterogeneous antibody population. In spite of this, however, we think that most of the antibody molecules in the first and last eluates differ from each other as indicated by their diverse properties. The 4-hour PCA activity may be due to 7S γ_1 antibody which so far is the only mouse immunoglobulin known to have homocytotropic activity (Nussenzweig *et al.*, 1964; Barth and Fahey, 1965). The 72-hour PCA activity, which attains very high levels in sera of *T. spiralis* infected mice, may be the same which occurs early in sera of mice receiving a single dose of antigen. The immunoglobulin class of this antibody is unknown; it may be either a new type of immunoglobulin similar to the human IgE described by Ishizaka and Ishizaka (1966) or a special part of the 7S γ_1 population as suggested by McCamish (1967).

It would be of considerable interest to know why a living parasite is such an efficient stimulus in inducing the production of reagin-like antibody. This phenomenon has been attributed to such factors as the nature of parasite antigens, the continuous presence of antigen and the location of the antigen in the intestinal tract. The nature of the parasite antigen does not fully explain this phenomenon since injecting parasite antigens induces the production of reagin-like antibody only at a low level (Table 1). It is possible that continuous stimulation by antigen is an important factor in inducing the production of reagin-like antibody even though repeated stimulation with *T. spiralis* antigen resulted in the disappearance of this antibody from serum. However, if the constant presence of antigen is an important factor for stimulating the production of antibody it would probably be so only in the beginning of the infection since the antibody could not be detected in some *T. spiralis* infected mice in which contact with antigen was continuously taking place. Therefore, the constant presence of antigen by itself is apparently not sufficient to keep the production of antibody at a high level. This is in agreement with previous observations showing that repeated contact with antigen not only failed to increase the antibody level but also accelerated the disappearance of the antibody from serum (Mota, 1963a, 1964, 1967). In apparent contrast with this, a second infection of mice with *T. spiralis* produced an anamnestic response with a sharp increase in the antibody level as shown in this paper and previously observed by Ogilvie (1966) in studying infections of rats with *Nippostrongylus brasiliensis* and by Zvaifler *et al.* (1967) in rabbits infected with *Schistosoma mansoni*. However, during artificial induction of reagin-like antibody with 'dead' antigen, the reagin-like antibody of rats immunized with a single dose of antigen did not always disappear from serum after one additional dose of antigen, but on the contrary the amount increased in some animals and only decreased and disappeared after additional doses (Mota, 1964). Besides, as shown here, mice receiving a second injection of *T. spiralis* 'dead' antigen also responded with an increase in the reagin-like antibody. A further explanation for the high level of reagin antibody induced by helminth infection is that the location of the parasites in the host may influence the type of antibody produced. This does not seem to be a decisive factor, however, since reagins are produced by a variety of helminths whose location in the host is as different as that of *S. mansoni* (Ogilvie, Smithers and Terry, 1966; Zvaifler *et al.*, 1967), *T. spiralis* (Sadun, Mota and Gore, 1968), *Nematospiroides dubius* (Dash, personal communication 1966) and *Dirofilaria uniformis* (Sadun *et al.*, 1967). Since infection with living parasites is such a potent inducer of reagin-like antibody, we thought it was possible that repeated infections would be required to bring down the antibody production by infected animals. Fig. 2 shows that six additional

infections were necessary to cause the disappearance of the reagin-like antibody from the serum of infected animals. Even at this time the antibody was still present at a low level in some of the animals. Thus, there may be only a quantitative difference in the production of reagin-like antibody by 'dead' antigen or by a living parasitic infection, the latter inducing a much higher and sustained production of the antibody. In both situations the production of reagin-like antibody seems to be stimulated after the initial contact with antigen and then gradually subsides. This may occur either in animals under continuous and long-lasting antigenic stimulus after a single infection with *T. spiralis* or in infected animals receiving additional antigenic stimulation on being re-infected. This is in agreement with the previous suggestion that antibody-forming cells are able to produce reagin-like antibody only after the initial contacts with antigen and that they lose this ability after prolonged contact with antigen (Mota, 1963a). However, the problem of why a living parasitic infection is so effective in inducing the production of reagin-like antibody is still unresolved.

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