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The Immunological Response of the Rat to Infection with *Taenia taeniaeformis*

V. SEQUENCE OF APPEARANCE OF PROTECTIVE IMMUNOGLOBULINS AND THE MECHANISM OF ACTION OF 7Sγ2a ANTIBODIES*

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Summary. Passive transfer of immunity to *Taenia taeniaeformis* was achieved with serum taken 14, 21, 49 and 63 days after infection. The protective capacity of serum collected at 14 and 21 days resided in the 7Sγ2 immunoglobulins and appeared to be particularly the result of 7Sγ2a antibody activity. However, as the infection progressed the range of chromatographic fractions showing protective capacity was extended to all those containing 7Sγ2 and 7Sγ1 immunoglobulins. Fractions enriched for γM did not confer protection.

Immune serum containing 7Sγ2a antibodies was able to kill developing parasites after they had left the intestine, and the hepatic postoncospheral forms retained their susceptibility to antibody over the first 5 days of growth. After that time they rapidly became insusceptible to antibody both *in vivo* and *in vitro*. Susceptibility to antibody-mediated attack was complement dependent. This appears to be the first time that complement has been demonstrated to play a role in immunity to a helminth infection *in vivo*. This finding is discussed in relation to the phenomenon of cestode parasite survival in immune animals.

INTRODUCTION

Experimental studies on acquired resistance to cysticercosis in laboratory and domesticated animals have provided ample evidence for the occurrence of protective serum antibodies (Miller and Gardiner, 1932; Campbell, 1938a, b; Blundell-Hasell, Gemmell and Macnamara, 1968). However, the mechanism and site of action of these antibodies on the invading organisms remain unknown and the means whereby established tissue cysticerci survive despite antibody attack have not been explored experimentally.

In our recent work we have therefore attempted to characterize some of the features of protective antibodies in order to clarify these important aspects of the host–parasite relationship in taeniid metacestode infections. When serum samples were taken from rats and mice on the 28th day of infection with *Taenia taeniaeformis*, a marked association was

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found between protective capacity and antibodies of certain well defined physicochemical characteristics (Leid and Williams, 1974a; Musoke and Williams, 1975a). Antigenically, these antibodies were recognizable as 7Sγ2a immunoglobulins in the rat and, tentatively, as 7Sγ1 in the mouse, but in neither instance were we able to detect the homologous skin-sensitizing activity which is manifested by antihapten antibodies in these classes (Morse, Bloch and Austen, 1968; Revoltella and Ovary, 1969; Binaghi, 1971). We suggested that the migrating oncosphere was attacked by protective antibodies acting in concert with reagins, the latter mediating local inflammatory reactions which influence the site and effectiveness of the response (Leid and Williams, 1974b, 1975).

In the present study, we have extended our observations on the types of antibody responsible for protection in the rat by examining immunoglobulin fractions from serum samples taken at intervals over the first 9 weeks of infection with *T. taeniaeformis*. Several workers have proposed that protective antibodies in serum may only be effective on the very early stages of the parasite at the intestinal level (Leonard and Leonard, 1941; Froyd and Round, 1960), and some evidence is available to suggest that heat-labile serum factors are involved in this resistance (Heath, 1971). We have therefore undertaken a series of experiments designed to characterize the mechanism of action of antibody in terms of the tissues in which the protective response occurs, the specific phases of the developing parasite which are vulnerable to immunological attack, and finally the role of complement in the process of destruction of the challenge organisms.

MATERIALS AND METHODS

Parasite

The strain of *T. taeniaeformis* used in these experiments was derived from gravid segments obtained from Mr C. E. Claggett in the Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, Maryland. The parasite was maintained following the method of Leid and Williams (1974a).

Experimental animals

Sprague–Dawley rats 28–42 days of age were purchased from Spartan Research Animals, Haslett, Michigan. They were given proprietary brand food and water *ad libitum.*

Preparation of immune sera

Rats were given 500 eggs of *T. taeniaeformis per os* and killed 14, 21, 49 or 63 days later using CO_2 vapour. Blood was collected from the thoracic cavity after severing the vessels anterior to the heart, allowed to clot at room temperature for 2–3 hours and left overnight at 4°. Serum samples were stored at $-20°$ without preservatives.

Passive immunization

Recipient animals received intraperitoneal inoculations of serum or immunoglobulin fractions which had been filtered using a 0·45 μm filter (Millipore, Bedford, Massachusetts). One millilitre of serum or chromatographic fractions was injected at the time of oral challenge with 400 eggs. After 3 weeks animals were killed with CO_2 vapour and the total number of cysticerci in each liver was determined. The results were analysed statistically using a modified Student's *t*-test.

Immunoelectrophoresis and double immunodiffusion

Immunoelectrophoresis (IEP) and double immunodiffusion (DID) were performed following the methods described by Leid and Williams (1974a).

Preparation of antisera to rat immunoglobulins

Antisera to whole rat serum and rat immunoglobulins were prepared according to the procedures described by Leid and Williams (1974a).

Chromatography

The globulins from sera collected as above were precipitated with 50 per cent saturated ammonium sulphate (SAS) (three times) and dialysed against phosphate-buffered saline (PBS) until free of sulphate ions.

The procedure for ion-exchange chromatography of rat immunoglobulins was similar to that described by Musoke and Williams (1975a). Stepwise elution was performed using sodium phosphate buffers in the following sequence: 0·05 M, pH 7·8; 0·01 M pH 7·8; 0·05 M, pH 5·8; 0·1 M, pH 5·8; and finally 2 M NaCl. All buffers were made 0·015 M in NaCl. The pooled fractions under each peak were concentrated back to the original volume of serum using polyethylene glycol.

Preparation of 7Sγ2a immunoglobulin fraction

In order to determine whether antibody activity is associated with 7Sγ2 immunoglobulins other than 7Sγ2a, advantage was taken of the recent observation of Nezlin, Krilov and Rokhlin (1973) on the susceptibility of 7Sγ2b and 7Sγ2c to tryptic digestion. DEAE–cellulose purified fractions of 7Sγ2 from 21-day immune serum were subjected to trypsin digestion (DCC-treated trypsin, Sigma Chemical Company) at 1:100 ratio to protein for 17 hours at 37° in 0·05 M Tris–HCl buffer pH 8·0. After addition of an equimolar amount of trypsin inhibitor (Soy-bean-TS, Sigma Chemical Company), the hydrolysates were fractionated on a 2·5 × 90 cm column of Sephadex G-200 using a 0·05 M Tris–HCl buffer in 0·28 M NaCl.

Column eluates were collected in 2·8-ml fractions and the optical density of each fraction was determined using a Perkin–Elmer Coleman 111 Spectrophotometer. Protein peaks were analysed by IEP and DID with rabbit anti-whole rat serum and guinea-pig anti-rat 7Sγ2. The trypsin resistant fraction which consisted of 7Sγ2a alone was made up to the original serum volume with PBS and used in passive transfer experiments.

Isolation of T. taeniaeformis *larvae*

In vitro hatching and activation of eggs of *T. taeniaeformis* was carried out following standard methods (Silverman, 1954; Rickard and Bell, 1971; Heath, 1973). However, activation rates were low and this procedure could not be used to obtain suspensions of organisms that had shed their oncospheral membranes. For this purpose we resorted to the recovery of organisms from the liver parenchyma of rats after oral infection with eggs. Ten thousand eggs of *T. taeniaeformis* were given *per os* and parasites were harvested after intervals of 1, 2, 4, 6, 8 and 10 days. The livers from each rat group were minced finely with scissors and 0·25 per cent trypsin solution was added at the rate of 50 ml/g of tissue. The supernatant fluid was collected after the larger fragments of liver tissue had settled and was centrifuged at 1000 ***g*** for 10 minutes. This sediment, consisting of free hepatic cells and

parasites (Fig. 1), was washed twice with Hanks's BSS (Grand Island Biological Company, New York) and suspended in the same medium.

For the liberation of parasites older than 6 days it was necessary to incorporate collagenase (Sigma Chemical Company) at a concentration of 10 mg/100 ml and calcium at 5 mg/100 ml in the digestion mixture. This additional enzyme was required because a host fibrous capsule formed around the developing cysticerci.

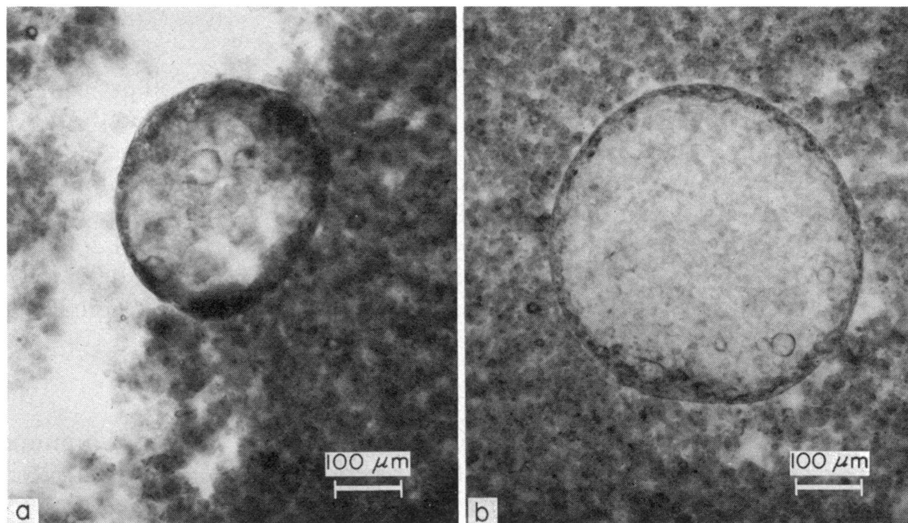

Fig. 1. (a) Six- and (b) 10-day-old larvae of *T. taeniaeformis* liberated from liver tissue by a combination of trypsin and collagenase digestion.

Isolation of cobra venom factor (CoF)

The anticomplementary factor from cobra venom (*Naja haje*, Sigma Chemical Company) was isolated following the method described by Ballow and Cochrane (1969). Five hundred milligrams of lyophilized cobra venom was dissolved in 40 ml of 0·01 phosphate buffer, pH 7·5. The solution was dialysed overnight before application to a DEAE–cellulose column (DE52, Whatman Company) 2·5 × 40 cm equilibrated with the same phosphate buffer.

The major toxic components passed through the column in the initial eluate and the remaining bound proteins were eluted using a linear 0·5 M NaCl gradient. Column eluates were collected in 2·8-ml fractions and the protein elution pattern was monitored by ultraviolet scanning at 280 nm (Fig. 2). Since most of the anticomplementary activity resides in the third protein peak (Maillard and Zarco, 1968), the eluates in this region were tested for their capacity to inhibit complement in normal rat serum using a haemolytic assay.

0·1 ml of each eluate was incubated with 0·5 ml of normal rat serum diluted 1:20 for 30 minutes at 37°. Sensitized sheep cells, prepared according to the method described by Kabat and Mayer (1971), were added at a final concentration of 2×10^8. The mixture was incubated for another 30 minutes at 37°. Two millilitres of cold PBS were added and the amount of haemolysis was determined by spectrophotometric analysis of the supernatant at 541 nm after centrifugation at 1000 ***g*** for 10 minutes. Tubes showing 50 per cent inhibition of haemolysis were pooled and concentrated. CoF units were determined using

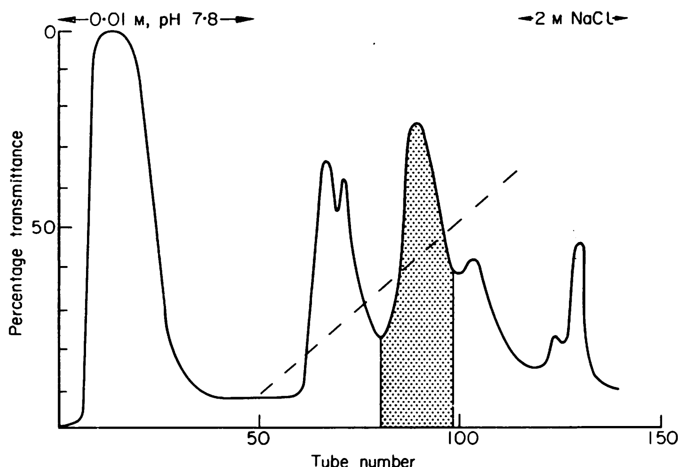

FIG. 2. Elution profile at 280 nm of cobra venom on DEAE–cellulose with phosphate buffer and 0·5 M NaCl gradient. The third peak (stippled area) was used to deplete complement levels in rats.

this haemolytic assay on 2-fold dilutions of the concentrated eluates. One unit was defined as that amount in 0·1 ml of CoF required to cause 50 per cent inhibition of lysis (Ballow and Cochrane, 1969). Further purification of the CoF was not attempted since active preparations produced no undesirable side effects in rats even after repeated injections.

Passive cutaneous anaphylaxis (PCA)

Homologous PCA tests were carried out on serum and immunoglobulin fractions following the procedure described by Leid and Williams (1974b).

RESULTS

Immunoglobulin preparations from 21-day immune serum containing all 7Sγ2 subclasses were used in passive transfer experiments. After oral challenge with 300 eggs the average number of parasites which developed in the liver of passively immunized rats was $1{\cdot}0 \pm 1{\cdot}7$ (s.d.), whereas in the controls the average was $19 \pm 6{\cdot}8$ ($P < 0{\cdot}001$). After tryptic digestion purified 7Sγ2a preparations were also used to passively immunize rats, and these and control animals were challenged orally with 500 eggs. The experimental rats were found to harbour an average of $0{\cdot}6 \pm 1{\cdot}6$ (s.d.) while the controls had $118 \pm 18{\cdot}9$ ($P < 0{\cdot}001$).

Having established a particular association of protective antibody activity with immunoglobulins of the 7Sγ2a type, we proceeded to examine the distribution of protective antibodies in other immunoglobulin classes in sera collected at intervals after infection.

Globulins from immune sera collected at 14, 21, 49 and 63 days after infection were precipitated with 50 per cent SAS (three times) and subjected to ion-exchange chromatography. Sequential stepwise elution was followed. The protein peaks were analysed for immunoglobulin types present in each protein pool by IEP. 7Sγ2a immunoglobulins were detected in 0·005 M phosphate buffer eluates while all the three subclasses of 7Sγ2 were present in all the remaining fractions. γM immunoglobulins were detected in the 2 M NaCl eluate. Reaginic antibodies were distributed in the 0·05 M and 0·1 M fractions from sera taken at 21 and 49 days post-infection.

Groups of 28-day-old rats were dosed with 400 eggs orally and then given 1-ml quantities of each chromatographic fraction intraperitoneally. Control animals received inoculations of normal rat serum. All groups of rats were killed 21 days later and the numbers of cysticerci developing in each group compared to the controls. The results, graphically represented in Fig. 3, indicated that as the infection progressed the spectrum of chromatographic fractions showing protective activity was extended to involve all those containing 7S immunoglobulins. However, eluates containing γM immunoglobulins were consistently not protective.

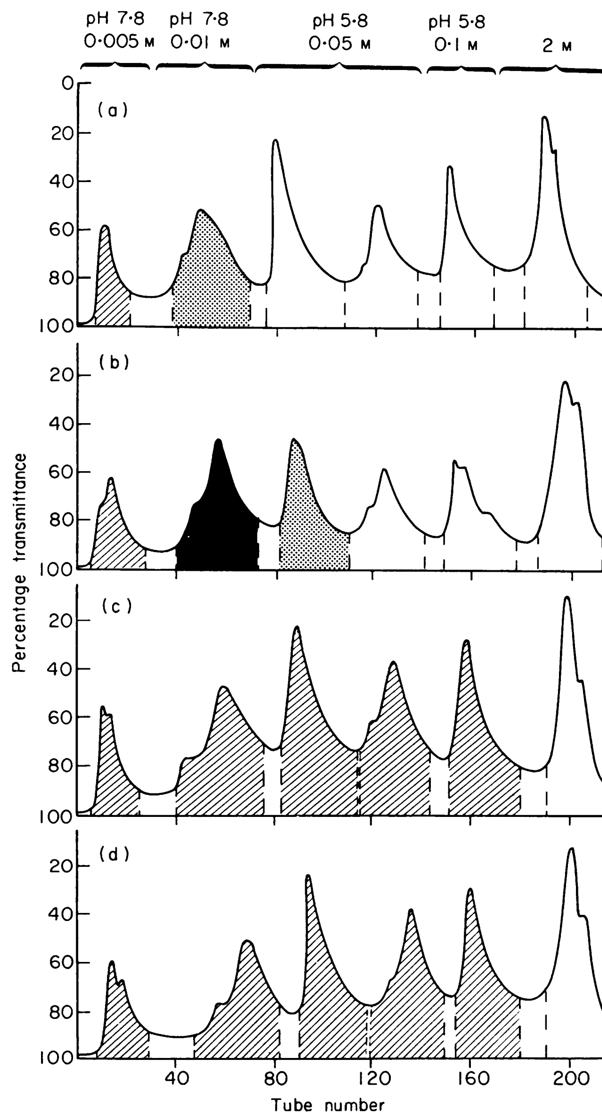

FIG. 3. Elution pattern at 280 nm of globulins (50 per cent $[NH_4]_2SO_4$) of immune rat sera collected 14, 21, 49 and 63 days after infection, on DEAE–cellulose with phosphate buffers and 2 M NaCl. All buffers were made 0·015 M in NaCl. Each protein peak was used in passive transfer experiments. Stippled areas indicate statistical significance of $P < 0{\cdot}05$; solid areas, $P < 0{\cdot}01$; hatched areas, $P < 0{\cdot}001$.

Experiments were then designed to establish whether or not these antibodies to *T. taeniaeformis* exerted their action only at the intestinal level. Twenty-eight-day immune serum was used in this experiment since the major protective activity was limited to one class of antibody at this time and high levels were present (Leid and Williams, 1974a). Rats were inoculated intravenously with 1 ml of 28-day immune serum. Thirty minutes later the rats were anaesthetized with a combination of ketamine hydrochloride intramuscularly and methoxyfluorane by inhalation and challenged via a mesenteric vein with 700 eggs. These had been exposed to artificial digestive juice *in vitro* and approximately a third of the eggs had hatched, although no more than 10 per cent had activated. Control rats were challenged with the same number of eggs *per os* after inoculations of either 1 ml of normal or immune serum intravenously. All rats were killed 21 days later and the number of cysticerci developing in the livers of each group are shown in Table 1.

TABLE 1

PASSIVE PROTECTIVE CAPACITY OF IMMUNE SERUM IN RECIPIENT RATS INOCULATED WITH 700 ARTIFICIALLY HATCHED AND ACTIVATED ONCOSPHERES OF *T. taeniaeformis* VIA A MESENTERIC VEIN

Treatment (i.v.)	Number of rats	Route of challenge	Mean number of cysts in liver ± s.d.	s.e.	*P* value
Normal serum	5	Intravenous	100·2 ± 32·5	14·5	—
Immune serum	6	Intravenous	0·0	0·0	< 0·001
Normal	5	oral	171·4 ± 17·5	7·8	—
Immune serum	6	oral	0·0	0·0	< 0·001

These results suggested that passive immunity could not be bypassed by avoiding intestinal migration and that protective antibodies could function outside the intestine. This finding was inconsistent with the proposals of Leonard and Leonard (1941) and Froyd and Round (1960) regarding the site of action of protective antibodies, but was consistent with the results of an experiment reported by Campbell (1938b). He had treated rats with immune serum at daily intervals after oral infection and shown that parasites within the liver remained fully susceptible to antibody for at least 4 days. He had speculated that the development of insusceptibility was due to formation of a fibrous host capsule which isolated the growing parasites from attack. We attempted to confirm his work on the development of insusceptibility in growing larvae and to establish if this change was derived from an isolating effect of the host capsule. Ten groups of rats were dosed with 500 eggs of *T. taeniaeformis* on day 0. Twenty-eight-day immune serum was administered to one group per day from day 0 to day 10. All rats were killed 21 days later. The results depicted in Fig. 4 showed that the effectiveness of antibody begins to wane by the 6th day.

Postoncospheral stages of *T. taeniaeformis* were liberated by enzymic digestion from livers of rats dosed orally with 10,000 eggs, at intervals of 1, 2, 4, 6, 8 and 10 days and exposed to normal or immune serum *in vitro*. The parasites were then injected via a mesenteric vein into recipient rats which were sacrificed 21 days later. The results, also depicted in Fig. 4, demonstrated that the development of insusceptibility to antibody is derived from inherent changes on the part of the parasites.

Attempts were then made to determine the role of complement in the process of immunological destruction of the parasites prior to day 6. In view of the fact that serum

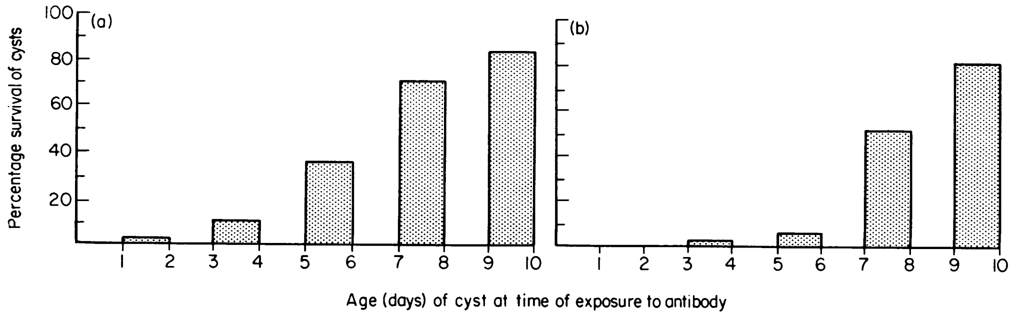

FIG. 4. Percentage survival of larvae of *Taenia taeniaeformis* of different ages after exposure (a) *in vivo* and (b) *in vitro* to immune serum containing 7Sγ2a antibodies.

complement levels can be effectively depleted using CoF for only 4–5 days (Maillard and Zarco, 1968), it was necessary to establish the optimum time at which to begin the CoF injections. Two groups of rats were inoculated with 1 ml of normal or immune serum intravenously and dosed with 2000 eggs of *T. taeniaeformis per os*. Twenty-four hours later the embryos were liberated from the liver tissue by tryptic digestion and injected via a mesenteric vein into normal recipients. The animals were killed 21 days later. The mean number of cysticerci in the rats receiving embryos from passively immunized rats was $21 \pm 3{\cdot}5$ while the control group had $25 \pm 10{\cdot}2$. These results indicated that there was a lag phase *in vivo* of at least 24 hours before a 1-ml dose of antibody resulted in death of the parasites.

In a subsequent experiment two groups of rats were dosed orally with 500 eggs of *T. taeniaeformis* followed by an intravenous inoculation of 1 ml of heat-inactivated 28-day immune serum. A control group received an equivalent amount of inactivated normal serum. Twenty-four hours later the control group plus one of the groups receiving inactivated immune serum began to receive intraperitoneal doses of 4 units per rat of CoF

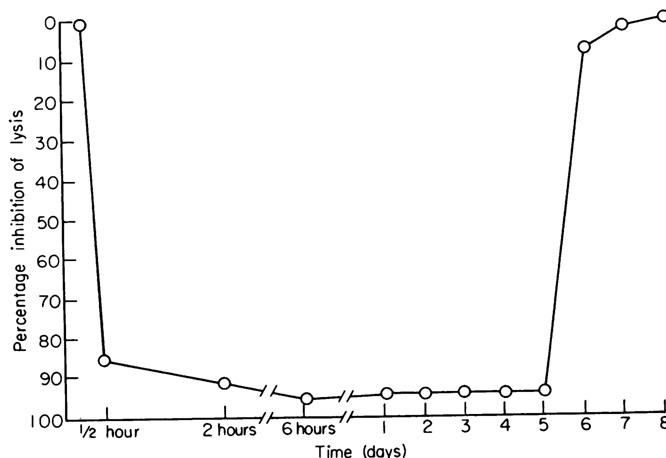

FIG. 5. Percentage inhibition of lytic complement in serum of rats inoculated with 4 units of CoF per rat every 6 hours for 5 days.

every 6 hours for 5 days. The daily levels of total complement were measured by the method described by Kabat and Mayer (1971) (Fig. 5). All rats were killed 21 days later and the mean number of larvae in each group is shown in Table 2. These results clearly demonstrate that an intact complement system is required for successful passive transfer of resistance to *T. taeniaeformis*.

TABLE 2

EFFECT OF DEPLETION OF COMPLEMENT (C3) ON THE ESTABLISHMENT OF *T. taeniaeformis* LARVAE IN RECIPIENT RATS INJECTED WITH INACTIVATED NORMAL OR IMMUNE SERUM. THE ANIMALS WERE CHALLENGED WITH 500 EGGS AND KILLED 21 DAYS LATER

Treatment	Number of rats	Average number of cysts in liver ± s.d.	s.e.	*P* value IRS × IRS-CoF
Inactivated normal serum + cobra venom (NRS-COF)	6	55·3 ± 14·2	5·8	—
Inactivated immune serum (IRS)	6	2·7 ± 2·2	0·9	—
Inactivated immune serum + cobra venom (IRS-CoF)	6	42·8 ± 13·0	5·3	< 0·001

DISCUSSION

Our results on the sequential appearance of protective antibodies in rats infected with *T. taeniaeformis* have confirmed the importance of 7Sγ2a immunoglobulins during the first 28 days of infection. Not only were the chromatographic fractions from 14- and 21-day serum samples which were enriched for 7Sγ2a the most effective for passive protection, but tryptic digestion of 7Sγ2b and 7Sγ2c from mixtures containing all three subclasses did not reduce the potency of these preparations. However, a remarkable extension of antibody activity to other chromatographic fractions was apparent with increasing time after infection until eventually all preparations containing 7S immunoglobulins conferred highly significant protection upon recipients.

These results suggest either that a diversity of antibody responses develops to the protective antigen(s) produced by the developing parasite or that the antigens produced by older stages of the parasite differ sufficiently to cause the appearance of antibodies of distinct immunoglobulin classes. The protective antigens in more mature parasites must nevertheless either be shared or cross-react with antigens of the early postoncospheral stages since the resistance mechanism is directed against these antibody labile forms.

None of the fractions enriched for γM immunoglobulin showed significant activity in any of our samples. This was particularly surprising since we have found that similar fractions prepared from serum of rats given surgical implants of mature metacestodes of *T. taeniaeformis* are highly effective in protecting recipients (Musoke and Williams, 1975b), whereas fractions enriched for 7Sγ2a were much less effective. It seems likely that these contrasting findings derive from the fact that antigens are presented to the immunological system of the host in a very different manner when live parasites are surgically implanted as opposed to their developing within the liver parenchyma.

With regard to the site at which protective antibodies exert their effect on the migrating

organisms, our results clearly demonstrate that this effector mechanism can destroy parasites outside of the intestinal environment and that postoncospheral developmental stages in the liver retain a high degree of susceptibility to antibody for approximately 5 days. We were unable to bypass the effects of circulating protective antibody by administration of challenge doses via the mesenteric vein and a clear pattern of gradually acquired invulnerability of hepatic parasites was demonstrated both *in vivo* and *in vitro*. These findings are comparable to the recent observations of Heath (1973) and Rickard (1974) indicating that metacestodes of *Taenia pisiformis* remain susceptible to the effects of immune serum for at least 1 week, and give little support to the idea of circulating antibody participation in an 'intestinal barrier' (Leonard and Leonard, 1941; Froyd and Round, 1960). This is not to say that there is no intestinal component to the resistance mechanism in cysticercosis, however, since we have been able to show that preparations of colostral γA from immune rats can be used to confer protection upon neonatal recipients, and that the action of γA is confined to the intestinal lumen (Musoke, Williams, Leid and Williams, 1975). It remains to be shown whether or not intestinal secretions containing γA contribute to the resistance shown by actively infected animals.

The observation that the effectiveness of protective antibody on the postoncospheral stages of *T. taeniaeformis* wanes as the parasites develop is in agreement with the results described by Campbell (1938b). However, he postulated that this decrease derived from formation of the fibrous host capsule around the parasite isolating the organism from antibody attack. The results of our experiments do not support this suggestion since larvae isolated at various stages *in vitro*, in the absence of the host capsule, also showed this shift toward the antibody-invulnerable phase, especially from day 6 onwards. It appears that the parasites themselves acquire some structural or metabolic characteristics which make them invulnerable to antibody-mediated attack. The observation that there is a 24-hour lag phase *in vivo* before protective antibody exerts its lethal effect is difficult to explain at this time but may indicate a requirement for other elements of the host defense system in destruction of the parasites.

The susceptibility of the early postoncospheral stages of *T. taeniaeformis* to antibody was shown to be dependent upon the integrity of the complement system in the host. Rats depleted of complement over this critical period were significantly deficient in their ability to destroy challenge organisms when given doses of immune serum which resulted in almost total destruction of all parasites in normal challenged animals. We believe that this represents the first instance of the effectiveness of antibody against a helminth being dependent upon complement *in vivo*, although other workers have used similar experimental approaches in their investigations (Jones and Ogilvie, 1971). The latter authors used CoF to deplete C3 levels in rats but were unable to implicate complement in the sequence of events which results in the expulsion of *Nippostrongylus brasiliensis* from the intestine. In our system the complement-dependent antibody-mediated attack on the early stages of *T. taeniaeformis* may be responsible for immobilization and destruction of the parasites by lytic effects and could result in the chemotactic attraction of either specific or nonspecific cellular components of the defense mechanism.

In an attempt to clarify the means whereby older parasites evade antibody attack, we have made repeated efforts to demonstrate the presence of amounts of haemolytic complement comparable to normal serum levels in the intracapsular fluid bathing the cysticerci of *T. taeniaeformis*. These efforts have been uniformly unsuccessful (unpublished observations). However, a great many other serum proteins were detectable in this fluid in

immunodiffusion tests. Although antigenic changes on the surface of the parasite (Varela-Diaz, Gemmell and Williams, 1972) or the masking effect resulting from adhesion of specific antibody (Rickard, 1974) have been postulated for the failure of rejection of cestode parasites in immune animals, it appears to us that anticomplementary factors produced by the metacestodes may be of prime importance in evading immunological damage. We have recently been able to demonstrate the release of anticomplementary substances by cysticerci of *T. taeniaeformis* both *in vitro* and *in vivo* (Hammerberg, Musoke, Hustead and Williams, in preparation) and are pursuing the biological significance of this finding in continuing studies in our laboratory.

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