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The Complement-Fixation Test in Hepatic Coccidiosis of Rabbits

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Summary. Antibodies to *Eimeria stiedae* were measured in rabbit serum by complement fixation. The titre rose to a maximum at about the 22nd day after infection, remained at this level for about 20 days and then declined. Antibodies were still detectable up to 160 days after infection.

Evidence of past or present slight *E. stiedae* infection was found in clinically normal rabbits whose sera fixed complement with *E. stiedae* antigens.

Challenge of rabbits which had recovered from a near-fatal infection had no effect upon the complement fixation titres of their sera.

The serum of a rabbit which had been injected with alum-precipitated antigen fixed complement with *E. stiedae* antigens. However, the animal was still susceptible to a superimposed oral infection which had the effect of further increasing the serum titre.

INTRODUCTION

Positive Wassermann tests in 'normal' rabbits have been related to Coccidial infections (Blumenthal, 1908; Kuczynski, 1921) but it was shown later that there was no connection between infection with *E. stiedae* and the results of the Wassermann test (Marcuse, 1922; Torres, 1924). The demonstration of resistance to re-infection with Coccidia stimulated several investigations into the possibility of the presence of circulating antibody in the sera of resistant animals, especially rabbits, and the complement-fixation test was used by Paterson, 1923; Chapman, 1929 and Bachman, 1930. Paterson used as antigens, normal saline, alcohol and carbol saline extracts of the livers of infected rabbits and the sera of known infected rabbits. His results were very variable but he concluded that the test was sufficiently reliable for diagnostic purposes. Chapman worked with rabbits infected with *E. perforans* and with antigens prepared from ground, dried oocysts or phenol-saline and alcohol extracts of infected intestine. With these antigens she found complement-fixation tests to be unreliable and precipitin tests uniformly negative. Bachman tested the sera of rabbits heavily infected with *E. stiedae* using alcohol, ether and saline extracts of infected livers but did not obtain consistent results.

Some properties of the precipitating antibodies present in the sera of rabbits infected with *E. stiedae* or injected with material prepared from this parasite have already been described but only a brief mention was made of antibodies demonstrable by complement fixation (Rose, 1959a). Also, the relationship between the precipitating antibodies in *E. stiedae* infections and the factors responsible for resistance to re-infection could not be demonstrated; it was hoped that a study of antibody levels throughout infection and after re-infection as measured by complement-fixation might give some indication of any such relationship.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS. Young rabbits, 4 weeks of age, were used for most of the experiments; a few older animals between 4 and 8 weeks of age were included. These animals were kept in isolation in metal cages on wire grid floors and the cages were steam-sterilized daily.

INFECTION OF ANIMALS. Oocysts were obtained from rabbit livers between 19 and 25 days after the infection of the host by macerating the livers in a Waring blender, screening out the coarser particles by passing the mixture through several sieves, the finest having a mesh diameter of 74 μ, and digesting the filtrate overnight at +4° with trypsin at pH 8. After separation of the liver cells by this digestion, centrifugation at low speeds will separate the heavy oocysts from the liver cell suspension. Oocysts were sporulated in 2.5 per cent potassium dichromate solution at room temperature.

Ten thousand freshly sporulated oocysts given to young 'coccidia-free' animals usually produced death within 18–25 days after infection (Group 1). Severe but not fatal infections were produced by giving 2500–5000 oocysts to similar rabbits (Group 2). Very large numbers of oocysts, 500,000–1,000,000, were given at the re-infection of recovered Group 2 animals. The pre-patent period in the infection is that interval between the entry of parasites into the host and the recovery of oocysts from the faeces.

At *post-mortem* examinations, the presence of macroscopic lesions caused by *E. stiedae* infection was noted and the contents of the gall bladder examined microscopically for oocysts.

ANTIGENS. Two types of antigen were used, one prepared from the exudate found in infected rabbit bile ducts (PBA) and one obtained by extracting crushed oocysts with 0.9 per cent saline solution (A). The antigens have been described (Rose, 1959a).

ANTISERA. Serum was obtained from rabbits before and at intervals after their infection and re-infection with oocysts of *E. stiedae*. Negative sera were obtained from a control adult animal, R70, which was kept in isolation and as free from contact with stray infection as possible and from a pool of serum taken from 14-day-old rabbits. Antisera were also prepared in rabbits by injecting them with alum-precipitated PBA antigen (Rose, 1959a).

COMPLEMENT-FIXATION TESTS. The 50 per cent end-point technique using 4 C'H50 units of complement was used in a procedure based on that described by Wadsworth, Maltaner and Maltaner (1931). The results were expressed as the ratio (IS+A)/IS of the titre of anti-serum in the presence of antigen (IS+A) to the titre of anti-serum alone (IS); antigen alone at the dilution used had no complement-fixing activity. The per cent haemolysis was measured in an EEL portable colorimeter using a green filter ($\lambda = 530$ mμ) and conversion factors (Kabat and Mayer, 1948) derived from the von Krogh (1916) equation, were used for calculating complement activity. The primary incubation period of antigen and antibody in the presence of complement was 18 hours at +4°.

RESULTS

COMPLEMENT-FIXING ANTIBODIES THROUGHOUT INFECTION

The results of complement-fixation tests on the sera of six rabbits (Group 1) after severe, eventually fatal infections with *E. stiedae* oocysts are shown in Fig. 1. The results of similar tests on the sera of five rabbits (Group 2) which had severe, non-fatal infections are shown in Fig. 2.

The pre-infection sera of two rabbits in Group 1 and of three in Group 2 showed slight complement fixation; the faeces of two of these rabbits (R14 and S) were examined and

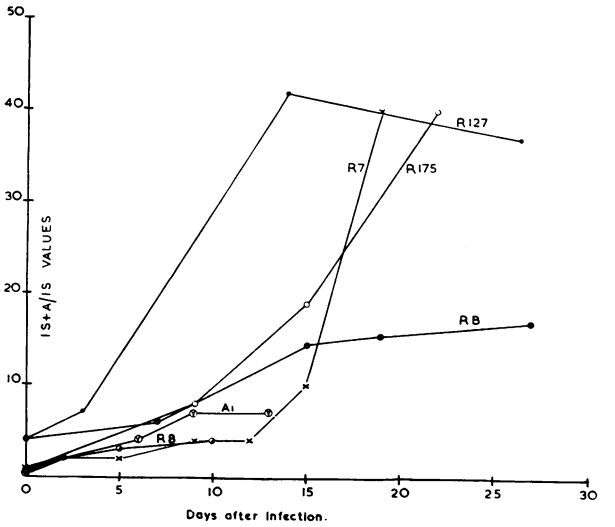

FIG. 1. Complement-fixation titres of sera of Group 1 rabbits with PBA as test antigen.

were found to contain oocysts. The faeces of R11 and R173 (no complement fixation by pre-infection sera) were negative for *E. stiedae* oocysts throughout the pre-patent period,

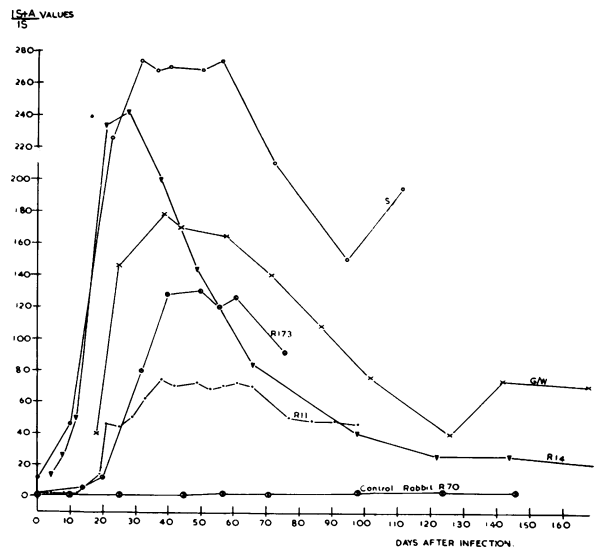

FIG. 2. Complement-fixation titres of the sera of Group 2 rabbits with PBA as test antigen.

indicating that there was no extraneous infection present before the administration of the experimental infection.

Slight increases in the complement-fixation titre were observed as early as 3–7 days after

infection (Fig. 1). When the infection was not fatal (Group 2, Fig. 2), the antibody levels rose fairly steeply to a peak value at between 30–40 days and, in two rabbits — R14 and G/W — whose sera were tested over a period of 200 and 228 days respectively, antibody remained demonstrable throughout the test period.

COMPLEMENT-FIXING ANTIBODIES IN THE SERA 'NORMAL' RABBITS

The results shown in Figs. 1 and 2 indicate that pre-infection sera sometimes gave positive complement-fixation tests and that, in two cases, this could be correlated with the presence of oocysts in the faeces before the expiry of the experimental pre-patent period. This suggested that the test might be used as a diagnostic aid. The results of tests on a small number of 'normal' young adult rabbits chosen at random are given in Table 1.

TABLE 1

RESULTS OF COMPLEMENT-FIXATION TESTS AND *post-mortem* EXAMINATIONS OF 'NORMAL' ADULT RABBITS

Rabbit	*Antigen*	*Antigen Dilution*	*IS+A IS*	*Presence of oocysts in gall bladder*	*Number of lesions on liver*
*X7	A	1 : 6	13	+++	1 large
*X8	A	1 : 6	3	—	1 very small
X9	A	1 : 6	16		
*X10	A	1 : 6	20	—	4
X11	A	1 : 6	13		
X12	B	1 : 60	3		
*14 day old rabbits (Pool)	A	1 : 6	1	—	—
*Control	A	1 : 6	1	—	—

* Post-mortem examinations available.

Post-mortem examinations were made on four of these animals. The sera from all the animals tested, with the exception of a pool from seven 14-day-old rabbits gave positive results. Oocysts were found in the gall bladder of only one of the rabbits examined; macroscopic lesions were found in the livers of three.

EFFECT OF CHALLENGE ON THE ANTIBODY LEVELS

Four rabbits which had survived a heavy initial infection with *E. stiedae* were used for testing the effect of challenge with 500,000–1,000,000 oocysts, on the complement-fixing antibodies. Two rabbits (G/W and S), were re-infected once, one (R134) was re-infected twice and one (R11) was re-infected four times. Results, together with the times of re-infection are given in Fig. 3.

Re-infection with large numbers of oocysts did not increase the complement-fixing activities of these sera. Similarly, there had been no evidence of the intensification of existing bands or the development of new precipitin bands when these same sera were tested by the Ouchterlony method (Rose, 1959a). None of the re-infected animals showed clinical signs of the disease, liver-function tests gave normal results and very few, if any, oocysts of *E. stiedae* were found in the faeces. No evidence of recent invasion by the

parasites was found at *post-mortem* examination of the livers of similar re-infected animals but isolated pockets of degenerate oocysts from the initial infection were present, surrounded by fibrous capsules.

FIXATION OF COMPLEMENT BY THE SERA OF RABBITS INJECTED WITH PARASITE MATERIAL

The injection of alum-precipitated antigens produced precipitating antibodies very similar to those obtained by oral infection with oocysts (Rose, 1959a). Antisera of this type fixed complement with both the immunizing antigen (BBA) and the oocyst antigen (A) giving titres of between 80 and 120.

Oral infection of a rabbit immunized with alum-precipitated antigen PBA and with a serum titre of 132, showed the same type of response as that of a litter-mate

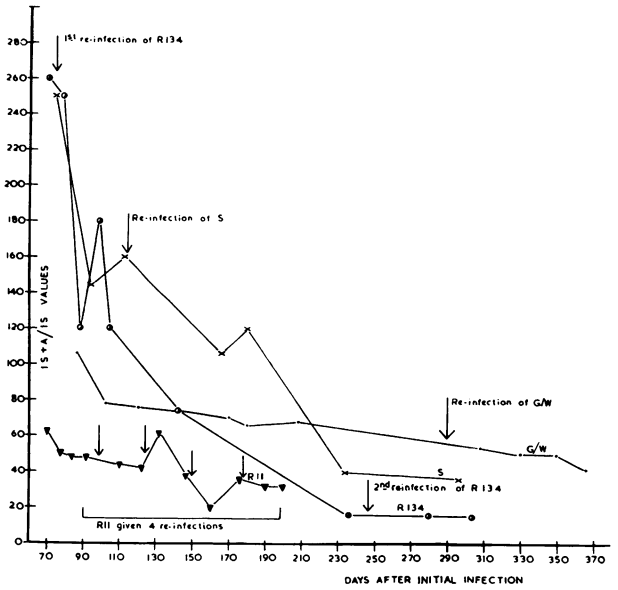

FIG. 3. Effect of challenge on complement-fixation titres.

infected at the same time but not previously immunized (Fig. 4). The already high complement-fixing power of the serum of the immunized rabbit was greatly increased after its oral infection and this increase was demonstrable with both test antigens. These results corroborate those obtained in agar precipitation tests where the superimposition of an oral infection on an immunized rabbit caused an intensification of the precipitin bands already existing (Rose 1959a) and indicate that a similar circulating antibody response is obtained by giving injections of *E. stiedae* material and by oral infection. In both immunized and non-immunized animals, however, an infection of roughly equal magnitude was subsequently induced by the oral administration of oocysts. The total oocyst output of the immunized rabbit was only slightly less than that of its non-immunized litter-mate and the ratio of liver to body weight was higher indicating that the infection was somewhat less severe.

DISCUSSION

The complement-fixation test was more sensitive than the gel-precipitation method already used, in that antibody could be detected over a longer period and was especially useful in showing the relative amounts of antibody present at different times after infection and re-infection.

Complement-fixation tests on the sera of rabbits given a single experimental infection with *E. stiedae* oocysts showed that a considerable amount of circulating antibody is developed during the infection and persists even after the completion of the patent period when all external evidence of the infection has disappeared and few lesions, if any, are visible in the liver. Reasonable precautions were taken to maintain all the experimental animals free from stray infections with oocysts so that it is likely that, in the main, the circulating antibodies demonstrated were those produced to the experimental infection.

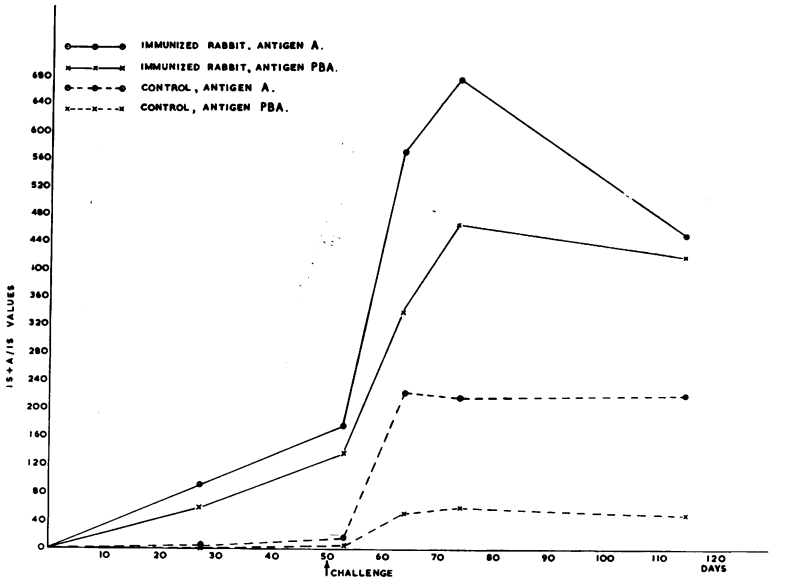

FIG. 4. Complement fixation by the sera of immunized and non-immunized rabbits with both oocyst and bile antigens.

Wherever complement-fixation occurred in clinically normal animals, careful *post-mortem* examination of the liver showed either the presence of, or lesions that had been caused by, a coccidial infection. In only one of these rabbits were oocysts found in the gall bladder and, probably this was the only rabbit which would be passing oocysts in the faeces. Thus faecal examinations for oocysts, the usual criterion for the presence of infection, are unreliable unless done daily over a considerable period of time and are of no use where the infection has been isolated by tissue reaction in the liver, or in the very early stages of the disease (up to 16 days after infection). Complement-fixation can therefore be useful in the selection of rabbits free from natural infection although the possibility of cross reactions where rabbits are infected with more than one species of Coccidium cannot be ruled out.

Administration of large numbers of oocysts to animals which had recovered from a

heavy experimental infection had no effect upon the level of circulating antibody. Similar doses given to apparently healthy rabbits with only slight infections acquired naturally and with low complement-fixation titres, resulted in a superimposed heavy infection together with a sharp and early rise in the complement-fixation titre. These findings corresponded to the effects of re-infection on the oocyst output; those that had recovered from heavy experimental infections showed little, if any, rise in their faecal oocyst output whereas rabbits with a very slight, naturally acquired infection passed many oocysts when given large infections (Rose, 1959b). The fate of the parasites given to the completely resistant animals is not known; they do not complete their life-cycle nor can they be detected in the liver at the times during which they would normally be there. If their invasion is 'blocked' at a very early stage, either in the duodenum, mesenteric lymph gland or liver, it is possible that there is not sufficient antigenic stimulus to produce a rise in antibody titre. The difference in response to re-infection of animals which have experienced a natural infection of a low order and those which have had large experimental infections is probably due to the quantitative difference between the initial infections. As a result of the slight natural infection the animal develops partial resistance and, when the large experimental infection is given, there may be a more rapid secondary type of response resulting in the high levels of circulating antibody (Fig. 2). A similar quantitative difference in response may account for the failure to protect animals from infection by injecting with parasite material.

The sera of rabbits injected with alum-precipitated material developed complement-fixing antibodies which reacted with both the immunizing antigen and an antigen (A) prepared from oocysts and qualitatively, these antibodies were the same as those found in orally infected rabbits. A superimposed oral infection further increased the level of complement-fixing antibodies. The animal which was injected with parasite material, however, was susceptible to the superimposed oral infection and developed a condition only slightly less acute than that of its control litter-mate, despite the relatively high level of serum antibody. The difficulty of obtaining and maintaining rabbits entirely free from natural infection during the immunizing period, coupled with a scarcity of antigenic material, prevented a thorough investigation of this problem. No evidence was obtained to show that the antibodies produced by injecting this particular antigen and (as these appear to be identical with those formed in response to an oral infection) those circulating antibodies which are detectable by gel-diffusion and complement-fixation methods in the sera of resistant animals, are responsible for the resistance of the host. It is possible that the factors concerned with the resistant state of the host are entirely or largely cell-bound and that the free, circulating antibodies which develop during the course of an infection play no active part in the defence mechanism of the host but merely accompany it. These antibodies, however, provide a useful guide to the existence and extent of infection and resistance. Recent work (Burns and Challey, 1959; Horton-Smith, Beattie and Long, 1961) on immunity to *E. tenella* infection in chickens has shown that resistance can be transferred from an infected caecum to its non-infected partner, but whether this is effected by circulating antibody or by some other factor has not yet been established.

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REFERENCES

BACHMAN, G. W. (1930). 'Serological studies in coccidiosis of rabbits.' *Amer. J. Hyg.*, **12**, 624-40.

BLUMENTHAL, F. (1908). 'Zur Serodiagnose der Syphilis. Diskussion' (Wassermann Reaktion und Kokzidiose der Kaninchen). *Berl. klin. Wschr.*, **45**, 618.

BURNS, W. C. and CHALLEY, J. R. (1959). 'Resistance of Birds to Challenge with *Eimeria tenella*.' *Exp. Parasitol.*, **8**, 515-26.

CHAPMAN, M. J. (1929). 'A study of coccidiosis in an isolated rabbit colony. The clinical symptoms, pathology, immunology and therapy.' *Amer. J. Hyg.*, **9**, 389-429.

HORTON-SMITH, C., BEATTIE, J. and LONG, P. L. (1960). 'Resistance to *Eimeria tenella* and its transference from one caecum to the other in individual fowls.' *Immunology*, **4**, 111-21.

KABAT, E. A. and MAYER, M. M. (1948). *Experimental Immunochemistry*. Charles C Thomas, Springfield, Illinois, U.S.A.

KUCZYNSKI, M. H. (1921). 'Ueber die Wassermannsche Reaktion beim Kaninchen.' *Berl. klin. Wschr.*, **58**, 125-6.

MARCUSE, K. (1922). 'Wassermannsche Reaktion und Kokzidiose beim Kaninchen.' *Zbl. Bakt. I. Abt. Orig.*, **87**, 355-62.

PATERSON, S. W. (1923). 'A complement-fixation test in coccidiosis of the rabbit.' *Brit. J. exp. Path.*, **4**, 1-4.

ROSE, M. E. (1959a). 'Serological reactions in *Eimeria stiedae* infection of the rabbit.' *Immunology*, **2**, 112-22.

ROSE, M. E. (1959b). 'A study of the life cycle of *Eimeria stiedae* (Lindemann, 1865) and the immunological response of the host.' Dissertation, University of Cambridge.

TORRES, C. M. (1924). 'Coccidiose, lésions de la moelle osseuse et réaction de Wassermann positive, non-spécifique, chez le lapin.' *C.R. Soc. Biol. (Paris)*, **91**, 986-7.

VON KROGH, M., (1916). 'Colloidal chemistry and immunology.' *J. infect. Dis.*, **19**, 452-77.

WADSWORTH, A., MALTANER, E. and MALTANER, F. (1931). 'The quantitative determination of the fixation of complement by immune serum and antigen.' *J. Immunol.*, **21**, 313-40.