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Autoantibody Production in Rabbits

III. THE EFFECT OF INFECTION WITH *EIMERIA STIEDAE* AND ITS RELATION TO NATURAL ANTIBODY

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Summary. The finding that the serum of apparently healthy rabbits fixed complement with rabbit liver and kidney has been confirmed. Experimental infection of rabbits with *Eimeria stiedae*, the cause of hepatic coccidiosis, led to a rise in the titre of serum complement-fixing factors. The rise was statistically significant 14, 21 and 28 days after infection. The factors were regarded as antibodies because they behaved as macroglobulins on diethylaminoethyl–cellulose chromatography and sucrose gradient centrifugation, and as autoantibodies because they fixed complement with the kidney of the rabbits in which they occurred. The antibody reacted with widely distributed antigen(s) with high activity in brain and low activity in skeletal muscle. The possibility that coccidial infection may be responsible for the natural autoantibody of rabbits is discussed.

INTRODUCTION

This paper describes the rise in titre of autoantibody in rabbits experimentally infected with *Eimeria stiedae*, the cause of hepatic coccidiosis. The observations were undertaken to investigate the origin of the natural autoantibody against rabbit liver and other tissues which occurs in the serum of apparently healthy rabbits (Kidd and Friedewald, 1942a, b). The association of autoantibodies with liver disease (Gajdusek, 1958; Mackay and Gajdusek, 1958) and infectious diseases including malaria and trypanosomiasis (Davis, 1944) suggested that infection of the liver by *E. stiedae* might cause autoantibody production. A similar suggestion was made by Blumenthal (1908).

E. stiedae is a sporozoan protozoon which lives during part of its life cycle in the epithelial cells lining the bile ducts of the rabbit. It multiplies extensively in these cells, destroying some and stimulating others to proliferation, causing the bile ducts to become enormously dilated and packed with parasite material. The resultant inflammatory reaction and fibrous capsule formation is associated with atrophy of the parenchymal liver cells. Because rabbits develop immunity after natural infection during the first 2 months of life young rabbits were taken. Littermate controls, which had not been experimentally infected, were used because natural autoantibody develops during the same period.

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MATERIALS AND METHODS

Infection of Rabbits

Four litters of cross-bred rabbits were used. Each litter was divided into equal groups, one was kept as a control and each member of the other was infected orally with sporulated *E. stiedae* oöcysts obtained in pure culture from the livers of infected rabbits. Rabbits 1, 2 and 3 of litter I were infected with 46,000 oöcysts when 4 weeks old and rabbits 7 and 8 of litter II at 6½ weeks. Rabbits 251 and 252 of litter III were infected with 23,000 oöcysts at 7 weeks and rabbits 256, 257 and 258 of litter IV at 3½ weeks. The rabbits were killed 1 month after infection, the livers examined macroscopically and kidney taken to serve as autoantigen. The livers of the severely affected rabbits were enlarged and covered with cysts; those of the mildly infected rabbits showed scattered small lesions.

Sera

Sera were heated at 56° before titration. Unless otherwise stated sera taken 4 weeks after infection were used. The sera of rabbits which had been isolated and given the anticoccidial drug nitrofurazone were also made available by the courtesy of Dr. I. Macdonald of Guy's Hospital (Macdonald, 1957).

Antigens

Homogenates of fresh or freshly frozen organs 1/10 (w/v) in 0·25 M sucrose were prepared using a Potter homogenizer with a Teflon pestle and were stored at −18°. The homogenate was diluted to the desired strength on the day of use with cold calcium magnesium saline buffered with sodium barbiturate (Oxoid barbitone CFT diluent tablets).

Polysaccharide Preparation and Haemagglutination

The methods of Broberger and Perlmann (1959) and Asherson and Broberger (1961) were used.

Trypsin Treatment of Antigen

A 1 in 5 (w/v) homogenate of rabbit liver, kidney and brain in 0·25 M sucrose was mixed with an equal volume of 0·04 M borate buffered saline, pH 7·7 with and without the addition of 2·5 mg./ml. crystalline trypsin (Armour). The solutions were dialysed against borate buffer for 1 hour at 37°.

Chloroform–Methanol Extraction of Antigen

The method of Asherson and Dumonde (1963) was followed. Alcohol extracts were prepared similarly.

Complement Fixation

Half-hour complement fixation followed by half-hour lysis at 37° was used and the results expressed as the reciprocal of the highest dilution giving 2 plus (50 per cent) haemolysis, estimated visually (Asherson and Dumonde, 1962). The symbol $\geqslant x$ was used to indicate that the antibody titre would have been x if the serum anti-complementary control had been negative. The data in Table 1 were obtained using 2·5 MHD_{50} of complement titrated in the presence of antigen. Rabbit kidney, rabbit liver and rat liver homogenates were used at dilutions of 1/40 to 1/80 (w/v) except for the kidney homogenates

from rabbits 251 and 252 which were anticomplementary and were used at a dilution of 1/120. The stock solution of Wassermann antigen (Difco Kolmer cardiolipin antigen) was diluted 1/50.

The other tests were carried out with 3 MHD_{100} titrated in the absence of antigen. Antibody and antigen controls were always included. The sera were heated at 56° for half an hour before testing. The eluates from the diethylaminoethyl–cellulose column were tested without heating.

Absorption of Sera

The procedure described by Asherson and Dumonde (1963) was employed.

Gel Diffusion

The method of Rose (1959) was used. Rat and rabbit liver extracts were prepared by homogenization in 3 volumes of saline followed by centrifugation at 144,700 *g* for 30 minutes.

Column Chromatography

The method previously described (Asherson and Dumonde, 1962) was used. The eluates at 0·01 M, pH 8; 0·1 M, pH 5·5; and 0·15 M, pH 4·3 sodium phosphate were collected in three separate fractions.

Sucrose Gradient Centrifugation

The method of Charlwood (1963) was used as described by Asherson and Dumonde (1963).

Statistical Analysis

The difference between the titres of the experimentally infected and control rabbits were compared within each litter by Student's *t*-test. For this purpose the rabbits were grouped in pairs using a table of random numbers. The pairs were, 1, 5; 2, 4; 3, 6; 7, 9; 8, 10; 252, 254; 256, 260; 257, 259; 258, 262.

RESULTS

RISE OF AUTOANTIBODY TITRE IN EXPERIMENTALLY INFECTED RABBITS

Ten rabbits from four litters were experimentally infected with *E. stiedae* oöcysts while other rabbits from the same litters were kept as controls. The titres of complement-fixing antibody of the sera of infected and control rabbits tested against their own kidney, the kidney of another rabbit and rat liver are given in Table 1. The titres against the three antigens were similar.

Most of the rabbits had initial titres under 16. After experimental infection, titres up to 512 occurred and the rise in titre in seven of the ten rabbits against homologous kidney was greater than 2 $\log_2$ units. None of the nine control rabbits showed a rise greater than 2 $\log_2$ units. The antibody titre in the infected rabbits against homologous rabbit kidney was 0·9, $\geqslant$2·0 and $\geqslant$2·0 $\log_2$ units higher than in the littermate control rabbits at 14, 21 and 28 days respectively. These results were statistically significant with P values of 0·02, $<$0·01 and $<$0·01. The titre of antibody against autologous rabbit kidney tended to be higher at 14 days and was significantly higher at 21 and 28 days. It was concluded that experimental infection with *E. stiedae* caused a rise in the titre of autoantibody.

TABLE 1

TITRE OF COMPLEMENT-FIXING ANTIBODY AGAINST AUTOLOGOUS AND HOMOLOGOUS RABBIT KIDNEY AND RAT LIVER IN THE SERA OF RABBITS EXPERIMENTALLY INFECTED WITH *Eimeria stiedae* AND THEIR LITTERMATE CONTROLS

Rabbit	*Week* 0			*Week* 1			*Week* 2			*Week* 3			*Week* 4		
	AK	*HK*	*RL*	*AK*	*HK*	*RL*	*AK*	*HK*	*RL*	*AK*	*HK*	*RL*	*AK*	*HK*	*RL*
Litter I															
1 infected*	–†	8	–	8	16	16	64	64	64	64	64	64	128	128	128
2 infected	–	8	–	–	16	32		64	64‡	–	–	–	–	–	–
3 infected	–	8	8	–	16	16		64	64‡	–	–	–	–	–	–
4 control	8	8	8	16	16	16	16	16	16	16	32	32	32	32	32
5 control	8	8	8	16	16	32	16	16	16	32	32	32	32	32	32
6 control	8	8	8	16	16	16	16	32	16	16	16	16	16	16	16
Litter II															
7 infected	32	16	16	16	16	16	64	32	64	512	128	128	512	256	256
8 infected	32	32	32	64	64	64	32	32	32	64	64	32	64	64	128
9 control	–	64	–	32	32	32	32	32	16	32	32	32	32	32	32
10 control	64	64	64	32	32	32	64	32	64	32	32	32	32	32	32
Litter III															
251 infected	8	8	–	16	16	–	64	64	–	64	64	–	64	64	–
252 infected	32	16	–	16	8	–	64	32	–	64	64	–	64	64	–
254 control	16	8	–	4	4	–	16	8	–	16	8	–	16	16	–
Litter IV															
256 infected	4	4	–	4	4	–	4	4	–	16	16	–	8	8	–
257 infected	4	4	–	8	4	–	8	8	–	16	32	–	16	16	–
258 infected	4	4	–	4	4	–	16	8	–	32	16	–	64	64	–
259 control	4	4	–	4	4	–	8	8	–	8	4	–	–	–	–
260 control	4	4	–	4	4	–	4	4	–	4	4	–	4	4	–
261 control	4	4	–	4	4	–	4	4	–	4	4	–	8	8	–

The sera were tested against homogenates of autologous rabbit kidney (AK), homologous rabbit kidney (HK), and rat liver (RL).

* Experimentally infected with *E. stiedae* oöcysts at week 0.

† The symbol – indicates no estimation available.

‡ Died of hepatic coccidiosis.

POOR CORRELATION BETWEEN THE TITRE OF AUTOANTIBODY AND LIVER CHANGES

There was little correlation between liver changes and the autoantibody titre. Although the titre in six rabbits with severe liver changes rose 2–4 $\log_2$ units, the titre in one infected rabbit with no liver changes and another with minimal changes rose 3–4 $\log_2$ units. The initial titre of 32 of one of the infected rabbits only rose 1 $\log_2$ unit despite moderately severe liver changes. The scanty liver lesions found in the control rabbits were attributed to natural coccidial infection, which most rabbits develop during the first 2 months of life.

THE RELATION OF HEPATIC COCCIDIOSIS TO NATURAL AUTOANTIBODY

A serum pool from rabbits 14 days old which lacked complement-fixing antibody to coccidial antigen (Rose, 1961) had an antibody titre against rabbit kidney of 4. A pool from slightly older rabbits with a low titre to coccidial antigen had an antibody titre against rabbit kidney of 16. Four rabbits, over a year old, which had been isolated and given the anti-coccidial drug nitrofurazone had titres against rabbit kidney of <4 and $\leqslant 4$. Four other rabbits which had been similarly treated and kept on a low protein diet had titres against rabbit kidney of <4, <4, $\leqslant 4$, and 32. The geometric mean titre was $\leqslant 4{\cdot}7$. The titre against rabbit kidney of eight other rabbits from the same institute, which had not been isolated or given drugs and had received a normal diet, ranged from

8 to 128 with a geometric mean of 21. Student's test showed that the difference between the means was significant ($P < 0{\cdot}05$ for 10 degrees of freedom).

Two pools of sera from rabbits injected with coccidial antigen which gave high titres of complement-fixing antibody to coccidial antigen both gave titres of 32 to rabbit kidney. This suggested that dead parasites did not enhance the level of natural autoantibody and that live organisms were required to raise the titre of autoantibody.

ANTIBODY TITRATION

Serum from rabbits 7 and 8 taken 1 month after infection reacted with rat liver and rabbit liver, kidney, heart, muscle, spleen, thymus and brain in titres from 32 to 256. Serum from two littermate controls, 9 and 10 gave titres from 16 to 32. No reaction was given to rabbit plasma diluted 1 in 10 taken from the same rabbit as the organs. The sera of other infected rabbits were shown to react with guinea-pig and human kidney. Block titration of the sera of five infected rabbits with rabbit liver and kidney revealed no prozones. The sera taken at 14, 21 and 22 days from the infected rabbit 7 gave a titre of 16 to 32 against the Wassermann antigen. None of the other nine sera from litters I and II reacted.

Absorption of sera from rabbits 1, 2 and 7 with washed deposits of foetal rabbit kidney showed that the antibody activity against rabbit liver and rabbit kidney was removed in parallel. When serum 7 was absorbed with either adult or foetal rabbit kidney, antibody activity against rabbit liver, rabbit kidney and the Wassermann antigen was removed in parallel.

The sera of four of the five experimentally infected rabbits which were tested by gel diffusion gave single lines with rabbit and rat liver extracts. The sera of five littermate controls were negative. Precipitating antibody to rabbit liver extracts first appeared 1–3 weeks after infection; that to rat liver at 2–4 weeks.

SUCROSE GRADIENT CENTRIFUGATION

Two sera with raised titres of autoantibody against rabbit liver and kidney obtained 1 month after coccidial infection were spun over a sucrose gradient. The distribution of antibody activity was similar to that found for the naturally occurring sheep-cell haemolysin known to be a macroglobulin (Table 2). There was no activity in the 7S globulin fraction. The activity in the macroglobulin fraction was destroyed by heating at 65° for 30 minutes.

DIETHYLAMINOETHYL–CELLULOSE CHROMATOGRAPHY

The sera of four normal rabbits and four rabbits experimentally infected with *E. stiedae* were fractionated by stepwise elution with sodium phosphate buffer. Typical results are given in Table 3 which shows that activity was recovered in the eluates at 0·1 M, pH 5·5 and 0·15 M, pH 4·3. No activity was found in the eluate at 0·01 M, pH 8.

HEAT STABILITY

Table 4 shows that the antibody activity against rabbit liver, rabbit kidney and rat liver dropped 4 $\log_2$ units or more after heating at 65° for half an hour. Heating at 56° did not affect the titre. It was concluded that the autoantibodies in the sera studied were heat-labile macroglobulins.

TABLE 2

TITRE OF COMPLEMENT-FIXING ANTIBODY AGAINST RABBIT LIVER AND KIDNEY IN FRACTIONS OBTAINED BY SUCROSE GRADIENT CENTRIFUGATION

	Fraction					
	1	2	3	4	5	6
Serum from infected rabbit 1						
Titre against rabbit liver	0	0	2	2	1	0
Titre against rabbit kidney	0	1	3	4	2	0
Serum from infected rabbit 7						
Titre against rabbit liver	0	1	3	4	0	0
Titre against rabbit kidney	1	2	4	5	0	0
Titre against Wassermann antigen	0	0	1	1	0	0
Titre of sheep haemolysin	0	0	1	3	0	0

Ten fractions, slightly greater than 1 ml. were collected. Fraction 1 was from the bottom of the tube. The figures represent the number of tubes giving significant complement fixation when the fractions were serially diluted. Fractions 7–10 were negative. The highest concentration of the marker 7S globulin was in tubes 7 and 8.

TABLE 3

ANTIBODY ACTIVITY OF ELUATES FROM DIETHYLAMINOETHYL-CELLULOSE CHROMATOGRAPHY OF SERA FROM RABBITS INFECTED WITH *Eimerica stiedae* AND THEIR CONTROLS

	Complement-fixation titre against			
	Rabbit liver	*Rabbit kidney*	*Rat liver*	*Wassermann antigen*
Serum A				
Whole serum*—normal†	256	256	–	–
Eluate 0·01 M, pH 8	<4	<4	–	–
Eluate 0·1 M, pH 5·5	⩾64	⩾64	–	–
Eluate 0·15 M, pH 4·3	32	64		
Serum B				
Whole serum—normal†	16	32	–	–
Eluate 0·01 M, pH 8	<4	<4	–	–
Eluate 0·1 M, pH 5·5	<4	⩽4	–	–
Eluate 0·15 M, pH 4·3	4	8		
Serum 7				
Whole serum—infected‡	64	64	64	16
Eluate 0·01 M, pH 8	<7	<7	<7	<7
Eluate 0·1 M, pH 5·5	<6	<6	<6	<6
Eluate 0·15 M, pH 4·3	36	72	72	18
Serum 251				
Whole serum—infected‡	256	256	256	–
Eluate 0·01 M, pH 8	<7	<7	<7	–
Eluate 0·1 M, pH 5·5	<7	<7	14	–
Eluate 0·15 M, pH 4·3	32	64	64	–

* Titre of serum applied to column.
† Serum from normal rabbits.
‡ Serum from rabbits 4 weeks after experimental infection with *E. stiedae*.

TABLE 4

THE EFFECT OF HEAT ON THE COMPLEMENT–FIXATION TITRE OF THE SERA OF RABBITS INFECTED WITH *Eimeria stiedae*

Serum		*Test antigen*		
		Rabbit liver	*Rabbit kidney*	*Rat liver*
1	Unheated	64	256	64
	Heated 56°	64	128	64
	Heated 65°	⩽4	⩽8	⩽4
7	Unheated	128	256	128
	Heated 56°	128	512	128
	Heated 65°	<4	4	<4
258	Unheated	32	256	16
	Heated 56°	32	128	16
	Heated 65°	<4	16	<4

NATURE OF THE ANTIGEN

Antigen activity was measured by the reciprocal of the highest dilution of organ homogenate giving significant complement fixation with a constant amount of antibody. Table 5 gives the activity of adult and foetal rabbit organs measured by five sera of infected rabbits and two control sera. The geometric mean titre of the activity of the adult

TABLE 5

ANTIGEN TITRATION. HIGHEST DILUTION OF ORGAN HOMOGENATES GIVING SIGNIFICANT COMPLEMENT FIXATION WITH SERA FROM INFECTED AND CONTROL RABBITS

Rabbit organ homogenate	*Sera*							*G.M.**
	7	8	1	2	252	9	10	
Liver	640	160	320	640	320	80	160	370
Foetal liver	320	160	160	640	320	160	320	280
Kidney	640	160	640	640	640	640	320	480
Foetal kidney	320	80	160	320	160	160	160	180
Lung	640	160	320	640	640	320	320	420
Foetal lung	320	–	320	160	640	–	640	320
Brain	640	320	640	640	640	320	640	530
Foetal brain	160	40	160	160	160	80	160	120
Muscle	⩾80	⩽10	40	80	40	10	⩾80	≏40
Foetal muscle	80	<10	40	80	80	40	⩾80	≏46
Heart	160	–	80	160	80	–	–	110
Foetal heart	160	80	⩾160	⩾160	⩾160	160	160	⩾140
Spleen	320	80	160	320	320	80	160	210
Placenta	160	⩽40	160	⩽80	320	⩽80	640	⩽120

Sera 7, 8, 1, 2 and 252 were from experimentally infected rabbits and used at concentrations of 1/32, 1/32, 1/20, 1/12 and 1/60 respectively. Sera 9 and 10 were from control rabbits and used at dilutions of 1/16.

* Geometric mean titre based on five sera from infected rabbits.

rabbit organs using the infected sera was brain 530, kidney 480, lung 420, liver 370, spleen 210, placenta ⩽120, heart 110 and muscle ≏40. The titre against foetal tissue was usually lower. The titre of foetal heart and muscle was slightly higher than the adult tissue probably because of the greater difficulty of homogenizing the adult tissue.

One-half to three-quarters of the activity of rabbit liver and kidney was lost on heating

to 56° for half an hour in 0·25 M sucrose. Kidney retained some activity at 70°. One of two batches of liver behaved similarly. Both antigens lost all activity when heated at 56° in saline.

The activity of rabbit liver, kidney and brain tested against the serum of infected rabbit 7 was not affected by trypsin (see 'Methods'). The serum of infected rabbits 7 and 252 (diluted 1/32 and 1/12 respectively) failed to react with the soluble cellular protein and soluble mitochondrial constituents released by ultrasonic disintegration of rabbit liver, kidney, heart and brain. These sera also failed to react with ethanol extracts of rabbit liver and kidney and serum 7 gave negative reactions with chloroform–methanol extracts of rabbit liver, kidney, brain and muscle and failed to agglutinate sheep red cells coated with a hot aqueous phenol extract of rabbit liver, kidney and brain. It was concluded that the antigen with which the complement-fixing antibody in the coccidial sera reacted was not soluble in 0·25 M sucrose and could not be obtained in an active form in chloroform–methanol and alcohol extracts.

DISCUSSION

The findings show that the sera of rabbits experimentally infected with *E. stiedae* during the first 7 weeks of life fix complement with rabbit kidney in significantly higher titres than their littermate controls. Kuczynski (1921) made the related observation that complement-fixing antibodies against extracts of syphilitic neonatal human liver were increased after experimental coccidial infection. Recently Augustin and Ridges (1963) have shown that turkeys infected with coccidia develop autoantibodies.

These complement-fixing factors were regarded as antibodies because they behaved as macroglobulins on diethylaminoethyl–cellulose chromatography and sucrose gradient centrifugation and were detected by classical immunological methods. They were regarded as autoantibodies because they reacted with the kidney of the rabbits in which they occurred. The possibility that the antibody was reacting with disseminated coccidial antigen was excluded by the similar activity of antigen in adult and foetal rabbit tissues and rat liver.

The autoantibodies produced by infection with *E. stiedae* and the natural autoantibody of Kidd and Friedewald, which occurs in apparently healthy rabbits, resembled each other and differed from the autoantibodies produced by the injection of rat tissue into rabbits. The first group of autoantibodies behaved as macroglobulins and were usually heat labile at 65° while the autoantibody produced 1 month after the injection of rat tissue also contained a 7S component and was relatively heat stable at 65°. However, the sedimentation behaviour of the heat-stable natural autoantibodies which occurred in the sera of certain rabbits was not determined. Again the first group of autoantibodies reacted with an antigen occurring in many organs while the autoantibodies produced by the injection of rat tissue reacted both with a widely distributed and an organ-specific antigen (Asherson and Dumonde, 1962, 1963).

The occurrence of natural antibodies has been attributed either to genetic factors directly determining the production of antibody without the intervention of antigen (Landsteiner, 1945) or to exposure to antigen. Springer, Horton and Forbes (1959) and Ashford, Fairfield and Bain (1961) have produced evidence that the natural antibodies to red cells arise as a result of antigenic stimulation. Our observation that the titre of autoantibody in rabbits is increased by infection with *E. stiedae* suggests that natural autoantibody, like other natural antibodies, occurs as a result of antigenic stimulation.

Whether this antigenic stimulus is provided by natural infection with *E. stiedae* or by some other agent is uncertain. The finding of natural autoantibody in rabbits without macroscopic hepatic coccidiosis and of precipitin lines against rabbit and rat liver in the sera of experimentally infected but not of control rabbits indicated that *E. stiedae* was either not the cause or not the only cause of the natural autoantibody or that very mild infections were sufficient to cause autoantibody production. The low titre of natural autoantibody in seven of the eight rabbits kept on anticoccidial drugs was compatible with the view that infective agents were important in maintaining the titre of natural autoantibody.

The occurrence of autoantibody in hepatic coccidiosis is an example of autoantibody production after infection. This has been noted in syphilis, malaria, leprosy, infectious hepatitis and yellow fever (Davis, 1944; Hughes, 1933). In the rabbit trypanosomiasis and syphilis cause autoantibody formation (Muschel, Simonton, Wells and Fife, 1961). It is not known whether coccidial infection causes autoantibody formation by a general stimulation of antibody production, by damaging the liver and liberating liver constituents, by providing carrier which renders a body constituent antigenic or by virtue of its own antigens. Models of the last three hypotheses are provided by the autoantibody formation which follows the injection of carbon tetrachloride into rats (Weir, 1961), the injection of pig serum and Wassermann antigen into rabbits (Sachs, Klopstock and Weil, 1925) and the injection of dead trypanosomes into rabbits (Landsteiner and van der Scheer, 1927).

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