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Characterization of the antibodies detected by the microscopic agglutination test for bovine leptospirosis

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SUMMARY

The nature of the antibodies detectable by the microscopic agglutination test for bovine leptospirosis was examined. Density gradient ultracentrifugation, gel filtration and disulphide-bond-reduction experiments indicated that antileptospiral agglutinating activity was present in both IgM and IgG immunoglobulin fractions. This was confirmed by selective precipitation of specific antibody classes and ion-exchange chromatography.

INTRODUCTION

Leptospira are often difficult to isolate from infected cattle and therefore diagnosis usually depends on the detection of specific antibodies. However, the efficiency of serological diagnosis obviously depends on the nature of the immunoglobulins active in the tests employed. The Rose Bengal plate test (RBPT) used in the detection of bovine brucellosis appears to be mediated solely by immunoglobulins of the IgG_1 sub-class (Corbel, 1972). It follows that the RBPT would be ineffective in detecting an early infection in which the immunoglobulins were predominantly IgM. In contrast, Duffus & Allan (1968) found that an indirect haemagglutination test was only capable of detecting the IgM response of chickens infected with *Salmonella gallinarum* although specific IgG antibodies were detectable by other means.

The microscopic agglutination (MA) test is the recognized standard serological test for bovine leptospirosis (World Health Organization, 1967). However, despite its extensive use, the antibodies detectable by the MA test have not been examined in bovine sera. A study to identify the agglutinins evoked in cattle by leptospira was therefore desirable in order to establish the phases of the immune response measured and, hence, the validity of the MA test in diagnosis.

MATERIALS AND METHODS

Serum samples

Bovine sera. Bovine sera from 3 animals each reacting to diagnostic titres ($\geqslant$ 1/100) with *Leptospira interrogans*, serotypes *icterohaemorrhagiae* and *canicola* were provided by the leptospirosis section at this laboratory. With the exception

of the ion-exchange chromatography studies, in which each of the samples was examined separately, pooled sera were examined.

Rabbit sera. Class-specific antiglobulins to bovine IgM and IgG had been previously prepared in New Zealand White rabbits using a similar method to that described by Negi, Myers & Segre (1971*a*). The antisera were rendered specific by absorption with purified bovine IgM or IgG polymerized with gluteraldehyde (Avrameas & Ternynck, 1969).

Microscopic agglutination test

The microscopic agglutination (MA) test, using living antigen suspension, was performed as described by Wolff (1954). The antigens used consisted of serotypes *icterohaemorrhagiae* and *canicola*, grown for 7 to 10 days at 30° C. in Korthof medium. Serum fractions were tested separately against each antigen.

Disulphide-bond reduction

Serum fractions were incubated at 37° C. for 60 min. with equal volumes of aqueous 0·2 M dithiothreitol (DTT, Hopkins & Williams). Serial doubling dilutions of the mixture were prepared in 0·15 M-NaCl and tested for agglutinating activity.

Selective removal of immunoglobulins

Class-specific rabbit antibovine globulin sera were used for the selective removal of IgM and IgG from whole bovine serum. The bovine serum was added to 4 volumes of the appropriate antiglobulin preparation and incubated for 16 hr. at 37° C. The precipitate was deposited by centrifugation at 10,000 ***g*** for 30 min. and the absorbed serum examined by immunoelectrophoresis and agglutination.

Density gradient ultracentrifugation

Gradients of $NaNO_3$ and KBr (Cowan & Trautman, 1965) were prepared according to Corbel (1972). Samples of 2·0 ml. of serum were centrifuged at a relative centrifugal velocity of 110,000 ***g*** for 24 hr. at 12° C. Serum fractions were aspirated in 0·5 ml. volumes and dialysed against 0·1 M phosphate buffer at pH 7·0.

Gel filtration

Gel filtration was performed in 700 mm. × 26 mm. columns of Sephadex G200 (Pharmacia). The gels were swollen in Tris-HCl buffer (1 M-NaCl; 0·1 M Tris-HCl, pH 8·0; 0·01 M-NaN_3) and equilibrated with 2 column volumes of the Tris-HCl eluant. Sera were dialysed overnight against the column buffer and 3·0 ml. volumes were applied using a three-way valve and eluted by upward development of the eluant at a flow rate of 20 ml. per hr. Column effluent was monitored for absorption at 280 nm. using a Uvicord ultraviolet absorptiometer (LKB Produkter). The fractions were collected automatically in 5 ml. volumes using an Ultra-Rac fraction collector (LKB Produkter), dialysed as described previously and concentrated to the original volume of serum with minicon macrosolute concentrators (Amicon Ltd).

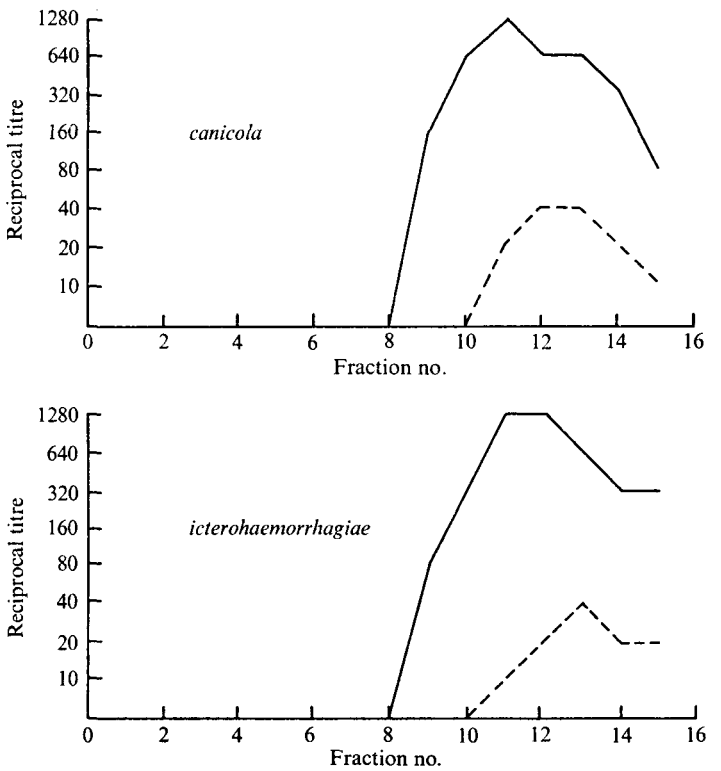

Fig. 1. Antileptospiral agglutinating activity of bovine serum separated by density gradient ultracentrifugation. ——, microscopic agglutination test (MA); – – – –, microscopic agglutination test after treatment with 0·2 M dithiothreitol (DTT).

Ion-exchange chromatography

Each of the serum samples was fractionated on QAE Sephadex A50 (Pharmacia) equilibrated with 0·1 M phosphate buffer at pH 7·0 in 300 mm. × 9 mm. columns. Serum was dialysed against the starting buffer and 2·0 ml. volumes added to the column which was developed by an increasing stepwise NaCl gradient in 0·01 M phosphate buffer at pH 7·0. Fractions of 2·0 ml. were collected and treated as described above.

Immunoelectrophoresis

Immunoelectrophoresis was performed on 82 mm. square slides in 1·5% ion agar in veronal buffer at pH 8·6 (I = 0·05) for 100 min. at a constant current of 15 mA. (Shreeve & Sojka, 1971).

RESULTS

Density gradient ultracentrifugation

The results of the density gradient ultracentrifugation are summarized in Fig. 1. Agglutinating activity to *icterohaemorrhagiae* and *canicola* was detected in both the fast-sedimenting and slowly sedimenting fractions. Reduction with DTT

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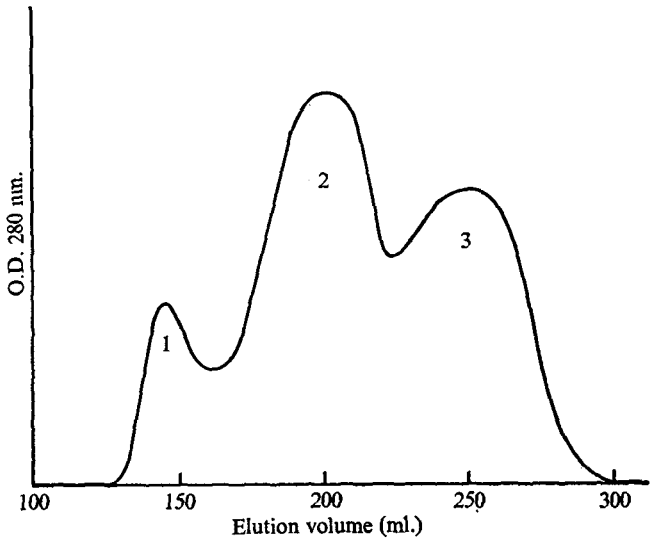

Fig. 2. Elution profile of bovine serum separated on Sephadex G200.

totally destroyed the agglutinating activity of the leading fast sedimenting fractions whereas the slower sedimenting fractions contained DTT-resistant immunoglobulins.

Gel filtration

The absorption profile of the serum separated on Sephadex G200 is shown in Fig. 2. Agglutinating activity to both serotypes was present in fractions corresponding to peak 1 and peak 2. Treatment with DTT showed that the agglutinins present in peak 1 were reduction-labile whilst DTT-stable agglutinins were eluted in peak 2 (Table 1). No agglutinating antibodies were detected in the fractions from peak 3.

Immunoglobulin inhibition

Immunoelectrophoresis of bovine serum absorbed with class-specific anti-globulin showed that the appropriate class of antibody had been successfully removed by precipitation. Microscopic agglutination tests after selective removal of IgM or IgG showed a significant reduction in the agglutination titre to *icterohaemorrhagiae* and *canicola* (Table 2). Absorption with normal rabbit serum had no effect on the titre.

Ion-exchange chromatography

Sera from each of the three animals were separated using the anionic exchange resin QAE Sephadex A50 and the eluted fractions tested for agglutinating activity against *icterohaemorrhagiae* and *canicola*. The absorption profile is shown in Fig. 3. No agglutinins were detectable in the fractions corresponding to peak 1, but DTT-resistant agglutinins were present in the fractions eluted in peak 2. The

Table 1. *Antileptospiral agglutinating activity of pooled bovine serum fractions separated by gel filtration on Sephadex* G200

Fraction no.	Serotype: *icterohaemorrhagiae* MA	*icterohaemorrhagiae* DTT	*canicola* MA	*canicola* DTT
Whole	320	80	160	40
1–25	—	—	—	—
	PEAK 1			
26	—	—	—	—
27	—	—	—	—
28	20	—	—	—
29	20	10	—	—
30	80	10	40	—
31	40	—	20	—
32	40	—	10	—
33	20	—	20	—
	PEAK 2			
34	10	—	—	—
35	—	—	—	—
36	—	—	—	—
37	20	—	10	—
38	40	20	20	10
39	40	20	20	10
40	40	40	10	—
41	10	—	10	—
42	—	—	—	—
43	—	—	—	—
	PEAK 3			
44	—	—	—	—
45	—	—	—	—
46	—	—	—	—
47	—	—	—	—
48	—	—	—	—
49	—	—	—	—
50	—	—	—	—
51	—	—	—	—
52	—	—	—	—

Titres expressed as reciprocal of final dilution showing agglutination.

MA, microscopic agglutination test; DTT, microscopic agglutination test after treatment with 0·2 M dithiothreitol; —, < 10.

majority of the DTT-labile agglutinins were eluted in peak 3 although activity was noted in some of the fractions corresponding to peak 4. No agglutinins were detected in the fractions from peak 5. Agglutinins were active against both serotypes tested (Table 3).

Immunoelectrophoresis detected only IgG_2 in the fractions eluted in peak 1. The proteins eluted in peak 2 appeared to be mainly IgG_1 with small amounts of IgG_2. IgM appeared to be the major immunoglobulin of peak 3 although traces

Table 2. *Antileptospiral agglutinating activity of bovine serum after selective precipitation of immunoglobulin classes*

Precipitating agent	Serotypes	
	ictero-haemorrhagiae	*canicola*
Normal rabbit serum	320	160
Rabbit anti-bovine IgM	40	40
Rabbit anti-bovine IgG	80	40

Titres expressed as reciprocal of final dilution showing agglutination.

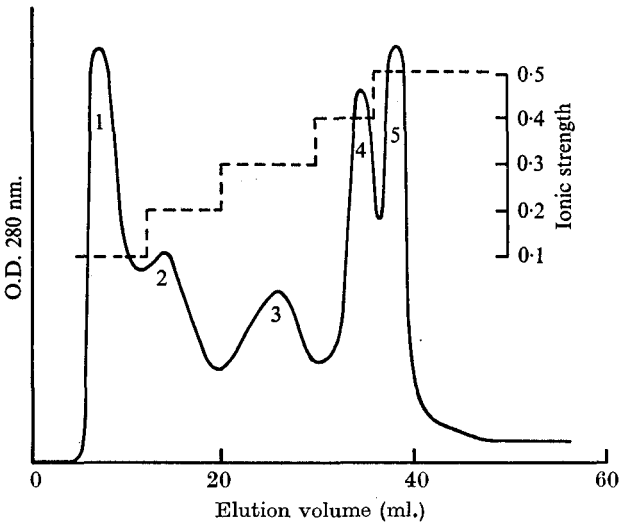

Fig. 3. Elution profile of bovine serum separated on QAE Sephadex A50.

of IgG_1 and possibly IgA were also present. The protein eluted in peaks 4 and 5 was more heterogeneous and contained only traces of γ-globulins.

DISCUSSION

There is little information concerning the nature of the immune response of cattle to leptospira infection. An examination of colostrum from infected dams suggested that while IgA exhibited neutralizing activity, agglutinating activity was associated only with IgM antibodies (Sascki & Arima, 1971). Specific IgG antibodies were not reported. Killed vaccines appeared to evoke insignificant agglutination titres in cattle, but appreciable levels of antileptospiral immunoglobulins of the IgM and IgG classes were detected using an antiglobulin test (Negi, Myers & Segre, 1971*b*). Furthermore, it was demonstrated that both these classes of antibody imparted some degree of protection to hamsters subsequently challenged with serotype *pomona*. In a recent review of the immunology of bovine leptospirosis, Hanson (1973) concluded that antigenic stimulation results initially

Table 3. *Antileptospiral agglutinating activity of bovine serum fractions separated by ion-exchange chromatography on QAE Sephadex A*50

Fraction no.	Peak no.	Cow 10 Ict MA	Cow 10 Ict DTT	Cow 10 Can MA	Cow 10 Can DTT	Cow 17 Ict MA	Cow 17 Ict DTT	Cow 17 Can MA	Cow 17 Can DTT	Cow 18 Ict MA	Cow 18 Ict DTT	Cow 18 Can MA	Cow 18 Can DTT
Whole		640	60	320	40	640	80	320	40	320	80	160	40
1	1	—	—	—	—	—	—	—	—	—	—	—	—
2		—	—	—	—	—	—	—	—	—	—	—	—
3		—	—	—	—	—	—	—	—	—	—	—	—
4		—	—	—	—	—	—	—	—	—	—	—	—
5		—	—	—	—	—	—	—	—	—	—	—	—
6	2	20	20	—	—	10	10	20	10	20	10	10	—
7		40	20	20	10	40	20	20	10	20	20	40	40
8		40	20	40	20	20	20	10	10	80	40	40	20
9		20	10	20	10	—	—	—	—	80	40	20	40
10	3	10	—	10	—	10	—	—	—	40	20	—	—
11		10	—	10	—	10	—	—	—	10	—	—	—
12		20	—	—	—	10	—	—	—	—	—	20	—
13		80	—	40	—	10	—	20	—	20	—	—	—
14		160	20	40	—	80	—	20	—	20	—	10	—
15		40	—	20	—	160	—	80	10	80	10	80	—
16	4	20	—	10	—	40	—	40	—	40	—	40	—
17		—	—	—	—	20	—	—	—	20	—	20	—
18		—	—	—	—	10	—	—	—	10	—	—	—
19	5	—	—	—	—	—	—	—	—	—	—	—	—
20		—	—	—	—	—	—	—	—	—	—	—	—
21		—	—	—	—	—	—	—	—	—	—	—	—
22		—	—	—	—	—	—	—	—	—	—	—	—

Ict, *icterohaemorrhagiae*; Can, *canicola*. For other notes see Table 1.

in a relatively short period of IgM production, associated with agglutination reactions, and later stimulation of neutralizing IgG antibodies detectable for a considerable time by hamster protection tests. If this is correct, the microscopic agglutination test might be expected to detect only the initial period of leptospira infection.

The association of agglutinating activity with IgM antibodies was confirmed in the present study. Fast-sedimenting immunoglobulins with agglutinating activity were demonstrated by density gradient centrifugation. The agglutinating activity in the fractions containing this class of immunoglobulin was destroyed by reduction with dithiothreitol (DTT) but similar activity was also demonstrated in the slow-sedimenting DTT-stable antibody, suggesting that both 19S (IgM) and 7S (IgG) agglutinins were present in the sera. Gel filtrations on Sephadex G200 confirmed that specific leptospiral activity was present in reduction-labile macroglobulin fractions and DTT-resistant fractions of lower molecular weight. Selective removal of IgM or IgG using class-specific antiglobulin sera caused a significant reduction in titre thereby indicating that both classes of antibody contributed to the antileptospiral agglutinating activity of the whole serum. This conclusion was strongly supported by ion-exchange chromatography on each of the serum

samples. Immunoelectrophoresis indicated that the γ-globulins were resolved into an IgG_2 fraction, a fraction containing IgG_1 and IgG_2, and a fraction comprising mainly IgM (and possibly some IgA). No agglutinating activity was detected in the IgG_2 fraction, but each of the IgG_1- and IgM-containing fractions were active in the MA test.

These results indicate that antileptospiral agglutinins are not restricted to the IgM class but that IgG_1 antibodies also exhibit agglutinating activity. Since the synthesis of IgG is said to lag behind that of IgM, although reaching higher concentrations and persisting appreciably longer than IgM, the microscopic agglutination test may detect residual antibodies over a far longer period than earlier studies of cattle sera suggest.

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