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Immunological response in cows to *Mycoplasma bovigenitalium*, strain 1836

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INTRODUCTION

The occurrence of contagious bovine pleuropneumonia (CBPP) in cattle in France (FAO, 1967) made it advisable to become familiar with diagnostic procedures for CBPP. CBPP reagents consisting of boiled *Mycoplasma mycoides* antigen, positive CBPP bovine serum and negative bovine serum were obtained from the Animal Health Research Laboratory, Parkville, Australia. Bovine serum samples were obtained from the Laboratory herds and from herds in various parts of England and Wales. Serum samples, taken from cattle affected by a wide range of pathological conditions, were also examined. The complement-fixation (CF) test used was that described by Campbell & Turner (1963). The results showed that one serum sample out of a total of 210 examined gave a significant reaction: at the 1/10 dilution almost complete fixation occurred, at the 1/20 dilution partial fixation and at 1/40 slight fixation. According to Campbell & Turner (1963), this reaction would have been classified as a weak positive which is usually associated with early cases of CBPP, chronic cases with small old sequestra or recovered cases with pleural adhesions or fibrosis.

The positive serum sample had been taken from a cow experimentally infected 3 weeks previously via the teat canal with *Mycoplasma bovigenitalium* (Stuart *et al.* 1963). A serological reaction between sera from *M. bovigenitalium* infected cows and *M. mycoides* antigen had been observed by Priestly using the agglutination test in Africa (R. H. Leach, personal communication). Roberts & Olesiuk (1967) showed that sera from chickens infected with *M. synoviae* reacted with *M. gallisepticum* antigens, whereas sera from chickens infected with *M. gallisepticum* did not react with *M. synoviae* antigens. Roberts & Olesiuk (1967) also showed that when *M. synoviae* was inoculated into birds, anti-γ-globulin activity was stimulated in the serum, and that *M. gallisepticum* antigens were sensitive to this anti-γ-globulin activity. Dr D. G. ff. Edward (personal communication) suggested that a relationship may exist between *M. bovigenitalium* and *M. mycoides* similar to that existing between *M. synoviae* and *M. gallisepticum*.

The aim of the present study was to investigate the immunological response of cattle to *M. bovigenitalium* and to study the serological relationship between *M. mycoides* and *M. bovigenitalium*.

MATERIALS AND METHODS

Mycoplasma bovigenitalium, strain 1836, was used, isolated from an outbreak of bovine mastitis (Stuart *et al.* 1963).

Media

The broth medium consisted of Difco PPLO broth, enriched with 10% inactivated horse serum, 1% yeast autolysate (Albimi), 0·2% dextrose, 1000 units penicillin G/ml., pH 7·8. Sloppy agar (agar concentration 0·04%) and agar plates were prepared with this medium using Oxoid Ionagar no. 2.

Animal inoculation

Six cows were used, varying from 2·5 to 6 years of age. Four were in full milk production while two were dry. Before inoculation all were tested serologically and culturally for evidence of infection with *M. bovigenitalium* and *M. mycoides*.

The six cows were inoculated with a broth culture of *M. bovigenitalium*, strain 1836. The four cows in milk were inoculated via the teat canal into the left fore and hind quarters, each quarter receiving $8{\cdot}1 \times 10^8$ organisms as judged by plate counts. One dry cow was inoculated via the nasal cavity and the other via the vagina, each cow receiving $8{\cdot}1 \times 10^8$ organisms.

Blood samples and milk samples were taken daily for 3 weeks, and thereafter at regular intervals. On each occasion a heparinized blood sample and a clotted blood sample were taken. Milk samples were taken on each occasion at approximately the same time, about 30 min. after morning milking. Attempts were made to isolate *Mycoplasma* from the heparinized blood and milk. Serum was prepared from the clotted blood and subjected to the serological techniques described below.

Isolation of mycoplasmas and their identification

The milk samples and heparinized blood samples were cultured in a similar manner. Two ml. of the sample was inoculated into sloppy agar and the sample cultured on PPLO agar. The agar plates were incubated in a moist atmosphere at 37° C. The sloppy agar cultures were incubated for 5–6 days, and then subcultured into sloppy agar and on agar plates.

Mycoplasma strains were cloned from single colonies, and were then identified using the growth inhibition test (Clyde, 1964). Broth cultures were plated on PPLO agar using cotton-wool swabs. Sterile 6 mm. antibiotic assay disks (W. and R. Balston Ltd.) were saturated with undiluted specific mycoplasma rabbit antiserum, dried at 37° C. and then stored at −20° C. until ready for use. The disks were placed on the inoculated agar surface, and the plates were incubated until the colonies became visible.

Antiserum production

Specific antiserum was prepared in rabbits against the 1836 strain of *M. bovigenitalium*. The organism was grown in broth, centrifuged at 10,000 rev./min. for 30 min., the supernatant fluid removed and the sediment resuspended in

physiological saline. The cells were washed six times and finally resuspended in physiological saline to a concentration equal to 2 × no. 10 Brown's opacity tube (Wellcome Research Laboratories). Rabbits were inoculated at weekly intervals for 6 weeks. Initially rabbits were inoculated subcutaneously at several sites with a total of 0·5 ml. antigen, homogenized in an equal amount of incomplete Freund adjuvant (Difco). The second and third inocula were similar but were given intramuscularly. The final three inocula consisted of antigen alone and were given intravenously. The rabbits were bled 1 week after the last injection.

Serological techniques

Stained serum plate agglutination (SA) test

A stained agglutination antigen was prepared using the 1836 strain. The organism was grown in broth and when sufficiently grown as judged by turbidity was centrifuged at 10,000 rev./min. The culture sediment was suspended in phosphate buffered saline (pH 7·0) containing merthiolate (1/10,000). The antigen was dispensed in glass tissue grinders and subsequently diluted to a concentration equal to 2 × no. 10 Brown's opacity tube. Rose Bengal was added to make a final concentration of 1/10,000, and the antigen was stored at 4° C. The SA test was carried out at room temperature; to one drop of serum on an enamel plate was added one drop of antigen; the two were mixed and the plate rotated. The test was read after 2 min., and a positive reaction was indicated by definite clumping. With positive reactors, twofold dilutions of the serum were prepared in physiological saline and the serum titres established.

Complement-fixation (CF) test

The CF test was used to detect the serological response in cows to *M. bovigenitalium*, strain 1836, and to determine whether a serological response occurred to *M. mycoides* antigen. The CF test of Campbell & Turner (1963) was used. Two 100 % units of complement were used with *M. mycoides* antigen and $1\frac{1}{4}$ units with *M. bovigenitalium*. Serial twofold dilutions of serum commencing at 1/10 were used, and the titration end-point was the serum dilution in the dilution following complete fixation.

The 1836 antigen was prepared by growing the mycoplasma in broth, centrifuging, washing three times in physiological saline and resuspending in physiological saline to 3 × no. 10 Brown's opacity tube. The suspension was boiled for 15 min., and 4 vol. of ether were then added to 1 vol. of the concentrated organisms. The mixture was shaken vigorously for 5 min., the supernatant ether layer removed, and the remainder placed in a water-bath at 60° C. and shaken to remove last traces of ether. The *M. mycoides* antigen was the boiled antigen mentioned previously, which had no ether treatment.

The CF test was also used to investigate the relationship between *M. mycoides* and *M. bovigenitalium*. Specific rabbit antiserum to *M. mycoides* was supplied by Dr R. H. Leach, Mycoplasma Reference Laboratory, Colindale, London. In this test $1\frac{1}{4}$ units of complement were used with both antigens.

Separation of immunoglobulins by density gradient centrifugation

One ml. of serum, diluted 1/2 in phosphate buffered saline (pH 7·2), was layered on a four-step linear sucrose gradient (10, 20, 30 and 40%, w/v) and centrifuged for 16 hr. at 35,000 rev./min. in a Spinco ultracentrifuge, using the SW 39 swinging bucket rotor. Ten serial fractions were collected through a hole pierced in the bottom of the centrifuge tube, using an MSE fraction collector. Fractions 2–3 are associated with IgM (19 S) activity and fractions 6–7 associated with IgG (7 S) activity (Kunkel, 1960; Coghlan & Weir, 1967).

Treatment of sera with 2-mercaptoethanol

Sera were treated with an equal volume of 0·2 M mercaptoethanol and incubated at 37° C. for 1 hr. IgM (19 S) is usually mercaptoethanol sensitive and IgG (7 S) is usually mercaptoethanol-resistant (Deutsch & Morton, 1958).

Treatment of sera with rivanol

Three parts of a 0·4% solution of rivanol (2-ethoxy, 6,9-diaminoacridine lactate) were added to one part of serum. The mixture was left at room temperature for 15 min., then centrifuged at 2000 rev./min. for 10 min. The supernatant was removed and tested serologically. Rivanol precipitates all the serum proteins except the γ-globulins (Patras & Stone, 1961).

RESULTS

Mastitis was produced in all the cows infected via the udder with clinical signs appearing within 24 hr. No clinical response was seen in the animals inoculated via the vagina and via the nasal cavity. The infected quarters became swollen and tender, while the uninfected quarters remained unchanged. The clinical signs and secretions of the affected quarters were characteristic of *M. bovigenitalium* mastitis (Stuart *et al.* 1963; Afshar, Stuart & Huck, 1966). By the 17th day after inoculation, the infected quarters of cows C and D had recovered from the infection and the appearance of the milk was normal. The left hind quarters of cows A and B did not recover and by the 19th week the quarters were dry. On the 18th day after inoculation, cow A was found to be very lame. The left hind stifle joint was found to be very painful and severely swollen, with the swelling extending up and down the limb. By the 20th day after inoculation the left carpal joint also was severely swollen; fluid was removed aseptically from the carpal joint and cultured for mycoplasma and other bacteria (Cowan & Steel, 1965). *Bacillus cereus* was isolated.

Milk samples were taken from the four cows for 18 weeks. *M. bovigenitalium*, strain 1836, was always isolated from the milk of the affected quarters. The mycoplasma could be isolated quite readily by culturing directly on PPLO agar. When mycoplasma colonies were not evident by this method, they were always isolated using sloppy agar medium. Mycoplasma were not isolated from the blood samples taken from the cows in the first 3 weeks after infection. *Bacillus*

cereus was, however, isolated from the blood on the ninth and eleventh days after inoculation. The *B. cereus* was isolated by way of the sloppy agar cultures.

The antibody response in three of the cows is shown graphically in Fig. 1. Cows A–D are the cows inoculated via the teat canal, the response in cow D was

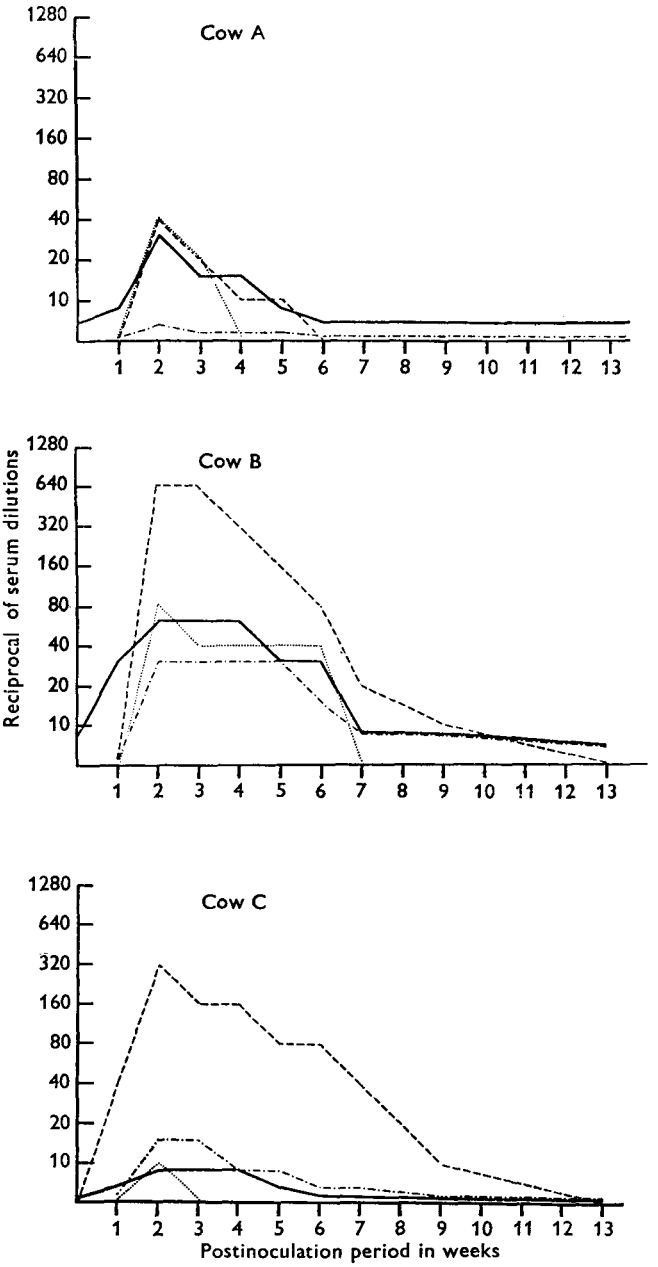

Fig. 1. Serological response in cows A, B and C to *M. bovigenitalium* strain 1836. — — —, 1836 CF reaction; · · · · · ·, CBPP CF reaction; ———, antiglobulin reaction; — · —, 1836 SA reaction.

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similar to that seen in cow C. The two cows inoculated via the vagina and nasal cavity did not produce any detectable antibody response as indicated by the CF and SA tests.

By the 5th day after inoculation an antibody response was detected in the two cows C and D using the SA test, although in cow D the reaction was delayed. By the 6th day, all the cows gave a positive reaction, but in cow B the reaction was again delayed, with 5 min. elapsing before clumping occurred. By the 7th day all four cows gave positive SA reactions, with the reactions occurring within 2 min. The highest SA titres observed in the cows varied from 1/4 in cows A and D to 1/16 in cow C and 1/32 in cow B; these titres occurred 2 weeks after infection and were followed by a gradual decline. The SA reaction appeared to be specific for *M. bovigenitalium*, strain 1836, infection. Blood samples taken from the laboratory herd were tested: only one serum sample out of a total of 58 gave a positive reaction. It was later found that this cow had been infected 6 years previously via the teat canal with strain 1836 (Stuart *et al.* 1963). Serum from this cow did not react with *M. mycoides* antigen in the CF test.

The homologous antibody response to *M. bovigenitalium*, as measured by the CF test, varied in the four cows. Maximum titres were reached 2 weeks after inoculation, and then the titres declined, and were less than 1/10 by the 6th week in cow A, by the 7th week in cow D, and by the 13th week in cows B and C.

The heterologous antibody response to *M. mycoides* varied considerably in the four cows, as did the anti-γ-globulin response. The maximum heterologous titres varied from 1/10 to 1/80 in the four cows; these titres were attained 2 weeks after inoculation. By the 3rd week after inoculation the titres were less than 1/10 in cows C and D. Titres greater than 1/10 persisted until the 4th week after inoculation in cow A and until the 7th week after inoculation in cow B. All four cows showed an anti-γ-globulin response as a result of inoculation with *M. bovigenitalium*. The anti-γ-globulin response followed a pattern similar to that of the heterologous antibody response to *M. mycoides* and the two appeared to be related. A titre of 1/64 was the highest anti-γ-globulin titre observed and this occurred in cow B, the cow that gave the highest heterologous antibody response to *M. mycoides* antigen. The lowest anti-γ-globulin titre of 1/8 occurred in cows C and D, the cows that also gave the lowest heterologous antibody responses to *M. mycoides* antigen.

With serum samples from cow B, collected on the 2nd, 3rd, 4th, 5th and 6th weeks after infection, only partial fixation occurred at the 1/10 and sometimes at the 1/20 serum dilution, in the CF test when titrated against 1836 antigen. During this period, high anti-γ-globulin titres were observed in the serum samples.

The rabbit antisera to *M. mycoides* and *M. bovigenitalium* were titrated for complement-fixing ability against *M. mycoides* and *M. bovigenitalium* antigens. There was no evidence of cross-reactivity among antigens and antisera.

The bovine and rabbit sera were subjected to sucrose density gradient centrifugation. In the serum samples collected from the four cows inoculated with the 1836 strain, all the homologous and heterologous antibody activity was detected

in the 19 S fractions. These included the SA reaction, anti-γ-globulin reactions, the CF heterologous reaction against *M. mycoides* antigen and the CF homologous reaction against *M. bovigenitalium* antigen. The treatment of the same sera with mercaptoethanol and rivanol confirmed these results. The SA and anti-γ-globulin reactions were sensitive to mercaptoethanol treatment. Treatment of the sera with rivanol removed the SA, anti-γ-globulin, CF homologous and CF heterologous activities. These results differed from those obtained with serum samples taken 2 years previously from a cow, 3 weeks after infection with the M 120 strain of *M. bovigenitalium* (Afshar *et al.* 1966): antibody activity was found both in the 7 S and 19 S fractions, with most of the activity in the 7 S fraction. The rivanol and mercaptoethanol treatments confirmed these results.

In the CBPP positive bovine serum and in the *M. bovigenitalium*, strain 1836, rabbit serum, antibody activity was associated with both the 19 S and 7 S fraction, with most of the activity associated with the 19 S fraction. The results were confirmed with mercaptoethanol and rivanol treatments.

DISCUSSION

A feature of the antibody response to the 1836 strain of *M. bovigenitalium* was the anti-γ-globulin response in the serum of the four cows infected via the udder. The response varied in the four cows, with the highest titre occurring in the cow which had the highest titre before inoculation. The results indicate that the anti-γ-globulin response was responsible for the heterologous response to *M. mycoides* antigen. Mycoplasma was not recovered from the blood during the three postinoculation weeks, nor was it recovered from the swollen joints of cow A. Lameness has been a feature of mycoplasma mastitis and mycoplasmas have been recovered from the joints (Jasper, Jain & Brazil, 1966). The significance of the *B. cereus* isolation from the blood and joints is not known, but *B. licheniformis* has been isolated from infectious synovitis in chickens (Roberts, 1964) and rod-shaped bodies have been seen in rheumatoid synovia (Roy & Ghadially, 1967). The high anti-γ-globulin activity in cow B may also have been responsible for the procomplementary activity of the serum.

M. bovigenitalium persisted in the udder for the duration of the experiment, which was 18 weeks. Latent infection is usually associated with persistence of serum antibodies. The maximum titres were reached 2 weeks after infection and then declined. The CF titres were less than 1/10 by the 13th week. The rapid decline in CF titres was striking, especially as the organism was still easily isolated from the milk. The CF test used in this experiment was not sensitive enough to detect low titres, but antibodies were detected using the SA test. The SA test was specific and was able to detect cows that were infected with the same organism 6 years previously.

In man and rabbits following immunization or infection with *M. pneumoniae*, the antibodies consisted of 19 S and 7 S (Schmidt, Lennette, Dennis & Gee, 1966; Fernald, Clyde & Denny, 1967). A factor which influences the kind of antibody produced is the nature of the antigen; lipopolysaccharide somatic antigens of entero-

bacteria stimulate IgM antibodies predominantly in man and rabbit (Pike, 1967). The inoculation of cows with *M. bovigenitalium*, strain 1836, produced IgM antibodies, whereas with the M 120 strain IgG was the predominant antibody. The CF test indicated that the two strains were similar. The two antigens used in the CF test were prepared differently, the 1836 strain had to be treated with ether before it was suitable, whereas the antigen of the M 120 strain was a washed suspension of the organism. The two strains appear to be antigenically distinct when compared using the growth inhibition test. These results are in agreement with those of Leach (1967), although he suggests that the use of a more potent antiserum would have shown an antigenic relationship between these strains by the growth inhibition test.

SUMMARY

Four cows were inoculated via the udder with the 1836 strain of *Mycoplasma bovigenitalium*. The serological response was investigated using the complement-fixation and serum plate agglutination tests. A heterologous response to *M. mycoides* was observed, associated with an increase in the anti-γ-globulin titres in the cow's serum. The heterologous response persisted in one cow for 6 weeks after infection. The nature of the immunoglobulin response was characterized by density gradient centrifugation, and by mercaptoethanol and rivanol treatment of the serum and was found to consist entirely of 19 S γ-globulins.

The *Mycoplasma* persisted in the udder for the 18 weeks duration of the experiment. The *Mycoplasma* was not isolated from the blood. One of the cows became lame with swollen joints; *Bacillus cereus* was isolated from the joints and blood.

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