A Monoclonal Antibody Specific for the A Antigen of *Brucella* spp.

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Two murine monoclonal antibodies of the IgG3 class have been isolated after immunization with *Brucella abortus*. An indirect immunofluorescence test was used to screen hybridoma supernatants and subsequently to determine the cross-reactivity of the monoclonal antibodies with other bacteria. One monoclonal antibody reacted with all the smooth *Brucella* biotypes tried and with *Yersinia enterocolitica* serogroup O:9, though not with rough *Br. ovis* or with strains of *Escherichia*, *Proteus*, *Salmonella*, *Pseudomonas*, *Francisella* and *Bordetella*. The other monoclonal antibody displayed a high degree of specificity for brucellae carrying the A lipopolysaccharide–protein surface antigen. The implications for the diagnosis of brucellosis are discussed.

INTRODUCTION

The considerable antigenic overlap between individual *Brucella* spp. has hindered the development of antibody reagents able to identify *Brucella* organisms to species and, ultimately, biotype level. One cause of serological cross-reactions is believed to be the lipopolysaccharide–protein complex bearing the A and M antigens in proportions varying with the species and biotype; this complex is present on the cell surface of all smooth brucellae (Diaz *et al.*, 1968*a, b*). The cross-reaction between *Brucella* spp. and *Yersinia enterocolitica* of serogroup O:9, first described by Ahvonen *et al.* (1969), has also been attributed to the A + M antigenic complex (Diaz *et al.*, 1970). However, Diaz & Bosseray (1974) later suggested that antibodies against the polysaccharide B antigen were involved. Lipopolysaccharide somatic antigens are also implicated in the cross-reaction between *Brucella* spp. and *Salmonella* serotypes of Kauffmann-White group N (Cioglia, 1950; Corbel, 1975).

The involvement of cell surface antigens in the cross-reactions within the *Brucella* genus and also between brucellae on the one hand and yersiniae and salmonellae on the other, is likely to hinder the development of specific fluorescent antibody reagents at least as much as it has the development of agglutinins and precipitins (Raybould, 1982). Early attempts to produce fluorescent antibodies against brucella organisms resulted in conjugates that were genus- but not species-specific (Biegeleisen & Moody, 1960). Corbel (1975) reported for a fluorescein-conjugated IgG to smooth *Br. abortus* that attempts to remove the cross-reactions with *Br. melitensis*, *Br. suis* and *Y. enterocolitica* O:9 by selective absorption with these organisms resulted in loss of all conjugate activity against brucellae and yersiniae. In contrast, the antiserum from which this conjugate was derived gave appreciable titres of *Br. abortus* specific agglutinins after cross-absorption with *Br. melitensis*. The fluorescent conjugate also stained group N salmonella organisms; but this reaction occurred only at low dilutions of conjugate, and could be removed by absorption with salmonella organisms without affecting the fluorescent staining of brucella or yersinia strains.

Monoclonal antibody technology (Kohler & Milstein, 1975) makes the detailed analysis of type-specific and cross-reacting antigens among *Brucella* spp. a feasible aim. The dissection of

Abbreviations: FITC, fluorescein isothiocyanate; IF, immunofluorescence.

the immune response in this manner can clearly lead to better understanding of the structure, localization and function of the antigen in different strains together with analysis of the cross-reactions in the animal immune response. In the work described below two murine monoclonal antibodies raised against *Br. abortus* were analysed for their cross-reactivity among different strains and biotypes of *Brucella* and among other bacteria using an indirect immunofluorescence (IF) assay.

METHODS

Antigens. *Brucella*, *Yersinia* and *Salmonella* strains of the stated biotype and serotype were kindly donated by Dr M. J. Corbel, of the MAFV Central Veterinary Laboratory, Weybridge, Surrey, UK. *Salmonella* strains, *Escherichia coli* O157 and *Proteus vulgaris* were grown overnight in nutrient broth at 37 °C, in shake flasks. *Bordetella pertussis* GL353 was grown as described by Morse & Morse (1976). Other bacteria were grown in a Difco GC medium base containing 10% (v/v) horse serum (Wellcome, no. 3) at 37 °C for 2 d.

To inactivate bacteria, formaldehyde was added to 1% (w/v) and suspensions were incubated overnight at 37 °C. The bacteria were then washed six times in phosphate-buffered saline (PBS: 0.1 M-NaCl, 20 mM-sodium phosphate buffer pH 7.7), and were finally suspended in PBS made 0.1% with respect to formaldehyde. Total counts were performed by phase-contrast microscopy. Suspensions were then diluted to 10^9 organisms ml⁻¹ and were stored at 8–10 °C. Working dilutions of bacteria were prepared freshly each day.

Production of monoclonal antibodies. Balb/c mice were immunized intraperitoneally (i.p.) with 10^8 *Br. abortus* 544 organisms in complete Freund's adjuvant, and again with 10^7 organisms two weeks later. Four months after the original immunization and 4 d prior to fusion the mice were boosted with 10^8 organisms in phosphate-buffered saline (PBS*: 0.15 M-NaCl, 3 mM-sodium phosphate buffer pH 7.3). Blood was collected and the 'hyperimmune' serum was separated and stored at -20 °C. The spleen cells from the immunized animals were fused at a ratio of 4:1 with P3-X63/Ag 8.653 mouse myeloma cells (Flow Laboratories) at 84% viability and in exponential growth. Hybrid cells were seeded at 10^6 cells per well in 24-well Costar plates in RPMI medium (Flow Laboratories) containing 20% (v/v) foetal calf serum (Northumbria Biologicals) supplemented with penicillin and streptomycin. Clones positive by immunofluorescence (IF) were selected and subcloned 35 d later. Clones 1B3 and 2C1 continued to secrete antibody after two subclonings at one cell per well on mouse spleen cell feeders in 96-well Costar plates and were selected for further analysis and expanded into 24-well Costar plates. Supernatants were stored at -20 °C.

Ascitic fluid was produced from the 2C1 clones. A Balb/c mouse was primed with an i.p. injection of 0.4 ml pristane (2,6,10,14-tetramethylpentadecane: Aldrich). After an interval of 10 d, the mouse received an i.p. injection of approximately 10^6 viable 2C1 hybridoma cells in 0.3 ml sterile PBS*. Five weeks later ascitic fluid was tapped from the peritoneum using a 21G-1.5" needle. Three further taps were removed at 2-d intervals. The ascitic fluid was centrifuged at 10000 g for 1 min; the supernatant was stored at -20 °C.

IF screening of hybridoma supernatants. Supernatants were assayed for anti-*Br. abortus* activity by indirect IF. 10^4 *Br. abortus* 544 organisms diluted from stock in distilled water were applied in 10 µl aliquots to multispot microscope slides [C. A. Hendley (Essex) Ltd] and dried at 65 °C for 20 min. The slides were fixed in acetone and stored. A 10 µl sample of the hybridoma supernatant to be tested was then added to each spot and the slides were incubated at room temperature overnight in a humidified chamber. The samples were removed with a Pasteur pipette and the slides were washed three times in 0.2 M-Tris/HCl, 0.2 M-NaCl, 0.05% Tween 20 pH 7.4 (TNT), once in 0.2 M-Tris/HCl, 0.2 M-NaCl pH 7.4 (TN) and once in distilled water, and were air dried. Then 5 µl FITC-conjugated sheep anti-mouse IgG, prepared essentially as earlier described for sheep anti-rabbit IgG (Phillips & Martin, 1982a, b), was added to each well at a concentration of 100 µg ml⁻¹ in normal serum buffer [1/6 (v/v) normal goat serum, 0.1% (w/v) Brij 35 (BDH) in PBS]. The slides were incubated in a humidified chamber for 1 h in the dark, then washed as before, dried and mounted in 10 mM-sodium bicarbonate buffer pH 8.2, 90% (v/v) glycerol for microscopy.

Negative (autofluorescence) controls were performed by substituting 1:100 normal mouse serum for the hybridoma supernatant. Positive controls were performed by substituting hyperimmune mouse serum at a dilution of 1:500.

IF testing for specificity. An indirect IF test was performed essentially as described above, but with the following modifications. 5×10^4 *Brucella* organisms or other test bacteria were dried on each well of the multispot slides, at 60 °C. The slides were fixed in ethanol. A 5 µl sample of supernatant, or ascites fluid, or hyperimmune mouse serum, diluted as specified in normal serum buffer, was added to the wells and incubated for 30 min. For autofluorescence controls, normal serum buffer was used alone. The slides were then washed three times in 0.1% Brij PBS and once in distilled water. The wells were then incubated with the FITC-conjugated sheep anti-mouse IgG for 30 min. After washing, the slides were mounted in the bleach retardant glycerol-based mountant Citifluor (Chemistry Department, The City University, London, UK).

Microscopy was performed using a Leitz fluorescence microscope (Dialux 20 fitted with a Ploemopak 2.4) at a magnification of $\times 1000$; fluorescence intensity of bacteria was scored subjectively on a scale from 0 (autofluorescence) to + + +.

Determination of antibody class. Antibody class was determined by ELISA (enzyme-linked immunosorbent assay) in a 96-well microtitre plate (Falcon). 10^6 *Br. abortus* 544 organisms were applied to each well in 50 μ l buffer and dried in a 65 °C oven. The cells were then fixed with methanol and dried. A 100 μ l sample of hybridoma supernatant was added to each well, and incubated at room temperature for 45 min. The wells were then washed once in TN, three times in TNT, once in TN, and once in distilled water, and were shaken dry. Then 100 μ l rabbit anti-mouse class-specific antibody (Miles) was added to each well at a starting dilution of 1:100 in 0.9% (w/v) NaCl containing 1:4 sheep normal serum, with doubling dilutions across the microtitre plate. The class-specific antibodies used were anti-IgG1, anti-IgG2A, anti-IgG2B, anti-IgG3, anti-kappa chain and anti-lambda chain. After incubating for 30 min and washing as before, sheep anti-rabbit horseradish peroxidase antibody (Miles) was applied at a dilution of 1:1000, and incubated for 30 min. The colour was then developed with *o*-phenylenediamine (BDH) at a concentration of 0.4 mg ml⁻¹ in the presence of 0.01% (v/v) hydrogen peroxide. The absorbance of the supernatants at 492 nm was measured on a Titretrek Multiskan Spectrophotometer.

RESULTS

In Table 1 the reaction of the monoclonal antibodies 1B3 and 2C1 with a range of *Brucella* strains and the *Y. enterocolitica* serogroup O:9 is compared with the reactivity of the hyperimmune mouse serum in the indirect IF test. Clearly, 1B3 exhibited a specificity as broad as that of the hyperimmune serum, reacting with all the smooth *Brucella* strains and with *Y. enterocolitica* but not with rough *Br. ovis*. In contrast, the 2C1 monoclonal antibody only reacted with *Brucella* biotypes believed to carry the A cell surface antigen; it did not react with *Y. enterocolitica* serogroup O:9. When a dilution series of the 2C1 ascites preparation was assayed, the reaction with *Br. abortus* biotype 7 and with *Br. suis* biotype 1 disappeared at lower dilutions than did the reactions with the other A⁺ *Brucella* strains. Results at two dilution points are shown in Table 1. Neither the hyperimmune serum nor these monoclonal antibodies reacted with the following bacteria: *Salmonella morehead*, *Salmonella* Br 1443, *Salmonella* Br 1775, *S. serenga*, *S. urbana*, *S. landau*, *Escherichia coli* O157, *Pseudomonas aeruginosa*, *Ps. multophila*, *Proteus vulgaris*, *Francisella tularensis* HN63 and *Bordetella pertussis* GL353.

When the immunoglobulin class of the 1B3 and 2C1 monoclonal preparations was investigated, both were found to possess an IgG3 heavy chain and kappa light chains.

Table 1. Specificity of mouse hyperimmune serum and monoclonal antibodies by IF

Organism			Score in IF test				
			Hyperimmune serum	Supernatant 1B3	Supernatant 2C1	2C1 ascites at dilution:	
Strain	Biotype	A and M antigens*				1:50	1:200
<i>Br. abortus</i> 544	1	A ⁺ M ⁻	+++	+++	+++	+++	+++
<i>Br. abortus</i> B3196	5	A ⁻ M ⁺	+++	+++	0	0	0
<i>Br. abortus</i> NCTC 10506	7	A ⁺ M ⁺	+++	+++	+++	++	+
<i>Br. melitensis</i> 16M	1	A ⁻ M ⁺	+++	+++	0	0	0
<i>Br. melitensis</i> H38	1	A ⁻ M ⁺	++	+++	0	0	0
<i>Br. suis</i> 1330	1	A ⁺ M ⁻	+++	++	++	+	0
<i>Br. suis</i> Thomsen	2	A ⁺ M ⁻	+++	+++	+++	+++	+++
<i>Br. suis</i> 686	3	A ⁺ M ⁻	+++	++	+++	+++	+++
<i>Br. neotomae</i> NCTC 10084		A ⁺ M ⁻	+++	+++	++	+++	+++
<i>Br. ovis</i> 63/290		A ⁻ M ⁻	0	0	0	0	0
Serogroup							
<i>Y. enterocolitica</i> 296/68	O:9		++	++	0	0	0

* See Brinley-Morgan & McCullough (1974).

DISCUSSION

The isolation of a monoclonal antibody (2C1) that has a high degree of specificity for A⁺ *Brucella* strains and yet is unreactive with *Y. enterocolitica* O:9 should be of some importance to laboratories concerned with the study and control of brucellosis. An epitope of the A antigen of the brucella surface lipopolysaccharide-protein complex is likely to be involved. On the one hand, highly specific monoclonal antibodies could be used to prepare more selective antigens as vaccines or for use in agglutination and precipitin tests of serological activity. Equally, such monoclonal reagents are likely to find direct application in the tests used in serological assays. Thus, monoclonal antibodies may be of use either for blocking antigens or as competitive inhibitors in assays for circulating antibody activity.

The advantages of IF over agglutination tests for detecting brucella organisms in clinical and animal samples, in terms of speed and of minimum numbers of bacteria detectable, have already been described (Moody *et al.*, 1961; Biegeleisen *et al.*, 1962). The rapidity of the indirect IF also gives it an advantage over agglutination for measuring anti-brucella antibody, although lower antibody titres are obtained using IF (Biegeleisen *et al.*, 1962). In view of the potential of IF methodology, the brief survey of the activity of mouse hyperimmune serum with non-brucella bacteria in the indirect IF test was performed early in this study. The cross-reaction results were similar to those previously reported for IF tests based on rabbit antibodies (Biegeleisen & Moody, 1960; Corbel, 1973; Corbel & Day, 1973; Corbel, 1975). Thus, it was concluded that the major disadvantage of using the mouse hyperimmune reagent for IF would be the cross-reaction with *Y. enterocolitica* O:9 and the lack of species specificity within the *Brucella* genus. In striving to produce monoclonal antibodies that would provide additional specificity in IF tests, an indirect IF technique was used to screen hybridoma supernatants, rather than one of the more usual ELISA techniques, in order to select directly for antibodies active against cell-surface antigens. This preliminary assessment of the specificity of the 2C1 monoclonal antibody for the A antigen serves to justify this experimental approach.

It is interesting to note that both antibodies produced are of the IgG3 subclass. This is a minor subclass of antibody in the mouse. A further monoclonal antibody from the same experiment proved to be of the IgM class but no antibodies of any other IgG subclass were detected. While clearly a larger panel of antibodies would need to be generated to form any firm conclusions, it is tempting to speculate that there may be an isotype preference for this particular antigen. The IgM antibodies are to be expected from any fusion experiment since some of the lymphocytes will not yet have undergone heavy chain switching, but two IgG3 antibodies are a comparatively rare phenomenon.

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