ELSEVIER

Veterinary Microbiology 60 (1998) 45–57

veterinary microbiology

Serological cross-reactivity between *Brucella abortus* and *Yersinia enterocolitica* 0:9: IV. Evaluation of the M- and C-epitope antibody response for the specific detection of *B. abortus* infections

Reinhold Kittelberger [a,*], Peter G. Bundesen [b], Axel Cloeckaert [c], Irene Greiser-Wilke [d], Jean-Jacques Letesson [e]

[a] *Central Animal Health Laboratory, Wallaceville Animal Research Centre, P.O. Box 40063, Upper Hutt, New Zealand*

[b] *AGEN Biomedical, 11 Durbell Street, Acacia Ridge, Qld. 4110, Australia*

[c] *Institut National de la Recherche Agronomique, Centre de Recherches de Tours, Laboratoire de Pathologie infectieuse et Immunologie, 37380 Nouzilly, France*

[d] *Institute for Virology, Hannover Veterinary School, Buenteweg 17, 30599 Hannover, Germany*

[e] *Facultés Universitaires Notre-Dame de la Paix, Faculté des Sciences, Unité d'Immunologie, 61 Rue de Bruxelles, 5000 Namur, Belgium*

Received 1 July 1997; accepted 28 October 1997

Abstract

Smooth lipopolysaccharides (SLPS) from *Brucella abortus* contain A-epitopes against which the majority of serum antibodies are directed during infections. SLPS from *Yersinia enterocolitica* 0:9 possesses identical epitopes, which are the cause for serological cross-reactivity. All *Brucella* spp. possess M- and C-epitopes which are not present in *Y. enterocolitica* 0:9. In order to examine the usefulness of these M- and C-epitopes for discrimatory serological testing, a panel of sera were used in this study, comprising sera from *Y. enterocolitica* 0:9-infected heifers, sera from *B. abortus*-infected cattle of comparable strength in the serological brucellosis tests to the sera from *Y. enterocolitica* 0:9-infected heifers, sera from *B. abortus*-infected bovines with strong serological reactions and sera from animals free from *B. abortus* or *Y. enterocolitica* infections. These sera were tested in blocking ELISAs with seven M- and one C-epitope-specific monoclonal

* Corresponding author. Tel.: +64-4-5274-482; fax: +64-4-5281-376; e-mail: kittelbergerr@wallaceville.mqm.govt.nz

PII S0378-1135(97)00202-2

antibodies in combination with SLPS from *B. melitensis* M16 high in M-epitopes as antigen. Strong *B. abortus* sera inhibited most strongly, while negative sera showed no or little inhibition. Sera with weak or intermediate titres blocked to a lower extent. Unexpectedly, the sera from *Y. enterocolitica* 0:9-infected heifers showed inhibition behaviour virtually identical to the comparable sera from *B. abortus* infected animals. Absorbing out of the A-epitope specific serum antibodies with either *Y. enterocolitica* 0:9 SLPS or with *Y. enterocolitica* 0:9 bacteria, indicated the presence of M- or C-epitope-specific serum antibodies in some sera from *B. abortus*-infected cattle but not in the sera from *Y. enterocolitica* 0:9-infected animals. These results demonstrate that the M- or C-epitope-specific antibody response in sera from *B. abortus* infected cattle is only of limited value for the serological discrimination between *B. abortus* and *Y. enterocolitica* 0:9 infections.

Keywords: *Brucella abortus*; *Yersinia enterocolitica*; Cross-reactivity; Monoclonal antibodies; Epitopes

1. Introduction

False positive reactions in the serological diagnosis of brucellosis in animals and humans are increasingly caused by infections with *Yersinia enterocolitica*, serotype 0:9 (Ahvonen et al., 1969; Benet et al., 1991; Corbel, 1985; Hilbink et al., 1995; Limet et al., 1992; MacMillan, 1990; Weynants et al., 1996). Such reactions are of particular concern for countries free from bovine brucellosis (MacDiarmid, 1994).

Conventional serological tests for *Brucella abortus* infections predominantly identify antibodies to the immuno-dominant SLPS of the bacterial cell envelope (Nielsen et al., 1989). Both, the *O*-chain parts of smooth lipopolysaccharide (SLPS) from *B. abortus* and from *Y. enterocolitica* 0:9 contain immuno-dominant homopolymers of 1,2-linked *N*-acylated 4-amino-4,6-dideoxy-α-D-mannopyranosyl residues (Caroff et al., 1984a), which explain this serological crossreactivity (Caroff et al., 1984b).

Much attention has been given to the development of methods for the differentiation of the serological response to *B. abortus* and to *Y. enterocolitica* 0:9. Differences in titres were found in cattle sera when tested in the Rose Bengal plate tests with *Yersinia* and *Brucella* antigens (Corbel and Cullen, 1970). Others used the presence of H antigens in *Y. enterocolitica* to distinguish from *Brucella* infections in an agglutination test (Mittal and Tizard, 1979). These tests were satisfactory in experimental infections but were less useful in evaluating field serum samples. Antigens other than SLPS were tried for the specific identification of serum antibodies against *B. abortus* (Cloeckaert et al., 1992b; Goldbaum et al., 1993; Limet et al., 1993; Kittelberger et al., 1995a) and against *Y. enterocolitica* (Kittelberger et al., 1995b; Weynants et al., 1996). The sensitivities of such tests, using protein antigens, were generally much lower than those using SLPS. However, some protein antigens appeared to be suitable for detecting animals with acute brucellosis (Baldi et al., 1996). It seems that, compared with the anti-SLPS response, the anti-protein antibody response in brucellosis is far too heterogenous and too delayed to be useful for serological diagnosis (Baldi et al., 1996; Cloeckaert et al., 1992b; Limet et al., 1993).

Specific detection of *B. abortus* infections could be based on the non-SLPS cellular immune response. Cell mediated immunity is used for the allergic diagnosis of bovine

brucellosis by performing an intradermal test with *Brucella* protein extracts (Bercovich et al., 1989; Bercovich and Ter Laak, 1990; Garin, 1993; MacDiarmid and Hellstrom, 1987). Despite being very useful at the herd level it is insensitive in individual animals. In vitro techniques for assessing the cellular immune response include the T-cell proliferation assay (Chukwu, 1985) and, more recently, the antigen-specific interferon gamma (IFN-γ) test (Weynants et al., 1995). Both have been used for the differentiation between *Y. enterocolitica* 0:9 and *B. abortus* infections. While the T-cell proliferation assay is not a practical routine diagnostic test, the IFN-γ test using Brucellergen as antigen lacks specificity (Kittelberger et al., 1997).

The SLPS of smooth *Brucella* spp. contain two distinct epitopes designated A and M, whose relative amounts vary among smooth *Brucella* strains (Bundle et al., 1987, 1989; Douglas and Palmer, 1988; Garin-Bastuji et al., 1990; Meikle et al., 1989). The A-epitope is identical to the already mentioned α-1-2-linked polymers of the pentasaccharide *N*-acetyl-4-amino-4,6-dideoxy-α-D-mannose and comprises four or five sugar units. This epitope is present in *Y. enterocolitica* 0:9 and in *Brucella* spp. The M-epitope seems to be specific for *Brucella* strains, which has been demonstrated by the use of M-epitope-specific Mabs (Bundesen et al., 1985; Bundle et al., 1984; Cloeckaert et al., 1992a; Greiser-Wilke and Moenning, 1987; Palmer and Douglas, 1989). In the M-epitope, some α-1-2-linkages in the pentasaccharide polymer are replaced by α-1-3-links, for example in the M-dominant *B. melitensis* M16 SLPS at every fifth position (Meikle et al., 1989). Other SLPS-epitopes that have been described are the common (C) epitope, which is strictly specific for smooth *Brucella* spp. and the C/Y epitope, which is common to *Brucella* spp. and *Y. enterocolitica* 0:9 (Weynants et al., 1997). While the structure of the C-epitope still awaits description, the C/Y-epitope appears to be a sequence of two to four units of α-1-2-linked polymers of the pentasaccharide *N*-acetyl-4-amino-4,6-dideoxy-α-D-mannose (Bundle et al., 1989).

The purpose of the present study was to evaluate the serum antibody response in *B. abortus* infected cattle against M- and C-epitopes by means of Mab-based blocking ELISA and by absorption ELISA, as a possible method for the identification of false positive reactors.

2. Materials and methods

2.1. Sera

Six sera (B1–B6) were obtained from cattle, experimentally infected with *B. abortus* strain 544 (Saegerman et al., 1994). Four sera (N1–N4) were from brucellosis-negative cattle from New Zealand (bovine brucellosis has been eradicated from New Zealand) and four sera (Y1–Y4) were from heifers experimentally infected with *Y. enterocolitica* 0:9 (Kittelberger et al., 1997).

All sera were tested in the YOP immunoblot (Kittelberger et al., 1995b) for the presence of antibodies against *Yersinia* outer proteins (YOP) from *Y. enterocolitica* 0:8 and *Y. pseudotuberculosis*.

2.2. Monoclonal antibodies

Mabs were produced and characterised as reported previously: Bm3-2 and Bm3-7, ascitic fluids (Bundle et al., 1989), Bruce 4, ascitic fluid (Bundesen et al., 1985); 2E11, ascitic fluid, 12G12 and 4F9, cell culture supernatants (Cloeckaert et al., 1992a); and Bm38 and Bm40, purified Mabs from cell culture supernatants (Greiser-Wilke and Moenning, 1987). They were further characterised in indirect ELISAs with SLPS from *B. abortus* (A-SLPS), *B. melitensis* (M-SLPS) and *Y. enterocolitica* 0:9 (Y-SLPS) (see Section 2.4).

2.3. Bacterial culture and preparation of SLPS

B. abortus B19 (biovar 1, vaccine strain, A-dominant) and *B. melitensis* M16, Rev. 1 (biovar 1, M-dominant) were cultured as reported elsewhere (Garin-Bastuji et al., 1990). *B. abortus* B19 SLPS (A-SLPS) and *B. melitensis* M16 (M-SLPS) were prepared by the hot aqueous phenol procedure of Leong et al. (1970).

Y. enterocolitica 0:9, a strain isolated from cattle in New Zealand, was grown as reported before (Kittelberger et al., 1995b). *Y. enterocolitica* 0:9 SLPS (Y-SLPS) was prepared by the method described by Caroff et al. (1984a,b).

2.4. SLPS ELISA

Nunc Maxisorp plates (96-well, Nunc, Kamstrup, Denmark) were coated with SLPS diluted in phosphate-buffered saline (PBS = 20 mM sodium phosphate/potassium hydrogen phosphate, pH 7.2, 0.15 M NaCl, 0.02% merthiolate) at 0.5 μg/well (100 μl/well) overnight at 4°C. The antigen was replaced with 200 μl/well PBS, containing 5% bovine serum albumin (PBS–BSA) and incubated for 60 min at 37°C. The plates were washed five times and then incubated for 120 min at 37°C with 100 μl/well of primary antibody. These were either bovine sera diluted in PBS–BSA in double dilution series, ranging from 1/10 to 1/10,240, or Mabs diluted in PBS–BSA in double dilution series, ranging from 1/10 to 1/2 million. Plates were washed five times and then incubated for 60 min at 37°C with 100 μl/well of peroxidase-conjugated recombinant protein G (Zymed, San Francisco, CA), diluted 1:5000 in PBS–BSA for the bovine serum ELISA, or with peroxidase-conjugated goat-anti mouse IgG (Fab) (Sigma, St. Louis, MO), diluted 1:2000 in PBS–BSA for the Mabs. After five washes, incubation was continued with TMB substrate at 100 μl/well (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 15 min at room temperature. The colour development was stopped by adding 100 μl of 10% hydrochloric acid per well. Absorbance was measured at 450 nm in a Bio-Tek Microplate Autoreader (Bio-Tek Instruments, Winooski, VT). Titres for the bovine sera were determined as dilutions at half of maximum absorbance at saturation of double dilution series.

2.5. Blocking ELISA

Plates were coated and blocked as for the SLPS ELISA. Incubations with bovine sera were performed for 60 min at 37°C, with 100 μl/well in double dilution series, ranging

from undiluted to 1/512 in PBS–BSA, followed by five washes. Incubations with Mabs were done next for 60 min at 37°C, with 100 μl/well, diluted in PBS–BSA at dilutions giving absorbance values of about one in the SLPS ELISA. The Mab dilutions were: 1/200 for 12G12, 1/5000 for Bm38, 1/10,000 for Bm40 and Bm3-2, 1/20,000 for Bruce 4, 1/100,000 for 2E11 and 1/1 million for Bm3-7. Subsequent steps in the assay were exactly as for the SLPS ELISA. Inhibitions were calculated for sera B1–B4 and Y1–Y4 individually, while for N1–N4 and B5 and B6 mean values were calculated. Results were expressed as percent inhibition of binding of the Mabs and were derived from the mean absorbance values for each sample by the following formula: percent inhibition = [1 − (mean absorbance value of test sample/mean absorbance value of the Mabs without serum)] × 100.

2.6. Absorption ELISA

Bovine sera at 1/50 dilutions in PBS–BSA were mixed with Y-SLPS solutions of 10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001 mg/ml and PBS–BSA (control) and incubated at 4°C for 17 h. Aliquots of 100 μl were taken and used in the A-, M- and Y-SLPS ELISAs as described under Section 2.4.

A series of ten-fold dilutions of *Y. enterocolitica* 0:9 in PBS–BSA was prepared from a suspension of wet bacterial pellet in two volumes of PBS–BSA. The strong positive sera B5 and B6, diluted 1/50 in PBS–BSA, were mixed with these suspensions, stirred for 17 h at 4°C and then centrifuged. Aliquots of 100 μl were run in the three SLPS ELISAs as described above.

2.7. Serological brucellosis tests

The complement fixation test (CFT), the serum agglutination test (SAT) and the *Brucella* card test (BCT) were carried out as described elsewhere (Alton et al., 1988).

3. Results

3.1. Characterisation of bovine sera

Serological reactions of the sera used in this study in the conventional tests (CFT, SAT and BCT), in three SLPS-indirect ELISAs and in the YOP blots are summarised in Table 1. Four of the six sera from *B. abortus*-infected cattle (B1–B4) were chosen because they were comparable in the brucellosis tests to the sera from *Y. enterocolitica* 0:9-infected heifers, exhibiting low to medium reactions in these tests. Two sera (B5 and B6) were strong positive sera. No major differences in titres for individual sera were found between the three indirect SLPS-ELISAs. None of the sera from *B. abortus* infected animals (B1–B6) and negative animals (N1–N4) showed staining in the YOP immunoblot, indicating that these animals were not infected with *Yersinia* spp. Three sera from *Y. enterocolitica* 0:9 infected heifers were positive in the YOP blot.

Table 1
Serological results of the panel of bovine sera used in this study

Serum	CFT titre[a]	SAT IU/ml	BCT grade	A-SLPS ELISA titre[b]	M-SLPS ELISA titre[b]	Y-SLPS ELISA titre[b]	YOP blot
B1	16	134	2	640	640	640	negative
B2	8	134	0	320	160	160	negative
B3	8	186	2	640	640	640	negative
B4	8	40	1	160	160	320	negative
B5	64	> 212	3	2560	2560	2560	negative
B6	64	> 212	3	2560	2560	5120	negative
Y1	16	80	1	320	320	320	negative
Y2	16	47	2	320	320	320	positive
Y3	16	17	1	320	320	320	positive
Y4	8	47	3	160	320	320	positive
N1	0	0	0	na	na	na	negative
N2	0	0	0	na	na	na	negative
N3	0	0	0	na	na	na	negative
N4	0	0	0	na	na	na	negative

[a] Titre = reciprocal dilution at any fixation.
[b] Titres are given as reciprocal dilutions.
na = not applicable.
B1–B6 = sera from *B. abortus*-infected animals, Y1–Y4 = sera from *Y. enteroclitica* 0:9-infected heifers, N1–N4 = sera from negative bovines. A-SLPS, M-SLPS and Y-SLPS = smooth lipopolysaccharides from *B. abortus* B19, *B. melitensis* M16 and *Y. enterocolitica* 0:9. CFT = complement fixation test, SAT = serum agglutination test, BCT = *Brucella* card test, YOP = *Yersinia* outer proteins.

3.2. Characterisation of monoclonal antibodies

Characteristics of the Mabs are listed in Table 2. All Mabs were characterised before (see references in Table 2) but the characterisation procedures varied between research groups, which either used ELISAs with bacteria- or with SLPS-coated plates. Furthermore, not all of the Mabs were characterised for their reactivity with *Y. enterocolitica* 0:9 SLPS. Additionally, characterisation on the basis of titres varies with the Mab preparation and with individual batches of ascitic fluids and cell culture supernatants. Therefore, the Mabs were re-characterised for this study under identical conditions, using indirect ELISAs with A-SLPS, M-SLPS and Y-SLPS as antigens. This resulted in a titration pattern for each Mab with the three antigens. Titres between Mabs could not be compared, due to the dependence of titres on the reagent type used (ascitic fluid, cell culture supernatant, purified antibody).

Four specificity pattern types were obtained (Fig. 1). Type I, represented by Mabs Bm3-2 and Bm3-7, showed high specificity for M-SLPS with no or very low antibody binding to A- and Y-SLPS. Mabs 12G12, 2E11, Bm38 and Bm40, which exhibited a type II pattern, reacted strongest with M-SLPS and almost equally strong with A-SLPS. They showed no or very low Y-SLPS reaction. A type III pattern was expressed by Mab Bruce 7, with strongest binding to A-SLPS, followed by weaker reaction with M-SLPS and still showing binding to Y-SLPS. Mab 4F9, which is an A-epitope specific antibody,

Table 2
Monoclonal antibodies used in this study

Mab	Class	Antigen used for immunisation	Specificity pattern type this study	Reported reactivity			Reported specificity	Reference
				A	M	Y		
Bm3-2	IgG3	*B. melitensis* 16 M	I	+	+	−	M	Bundle et al., 1989
Bm3-7	IgG2b	*B. melitensis* 16 M	I	+	+	−	M	Bundle et al., 1989
BM38	IgG1	*B. melitensis* 16 M	II	+	+	−	M	Greiser-Wilke and Moenning, 1987
BM40	IgG1	*B. melitensis* 16 M	II	+	+	−	M	Greiser-Wilke and Moenning, 1987
Bruce4	IgG3	*B. abortus* 19	III	+	+	−	M	Bundesen et al., 1985
2E11	IgG3	*B. melitensis* B 115	II	−	+	−	M	Cloeckaert et al., 1993
12G12	IgG1	*B. melitensis* B 115	II	+	+	−	C	Cloeckaert et al., 1993
04F9	IgG2a	*B. abortus*, biovar 3	IV	+	+	+	A	Cloeckaert et al., 1993

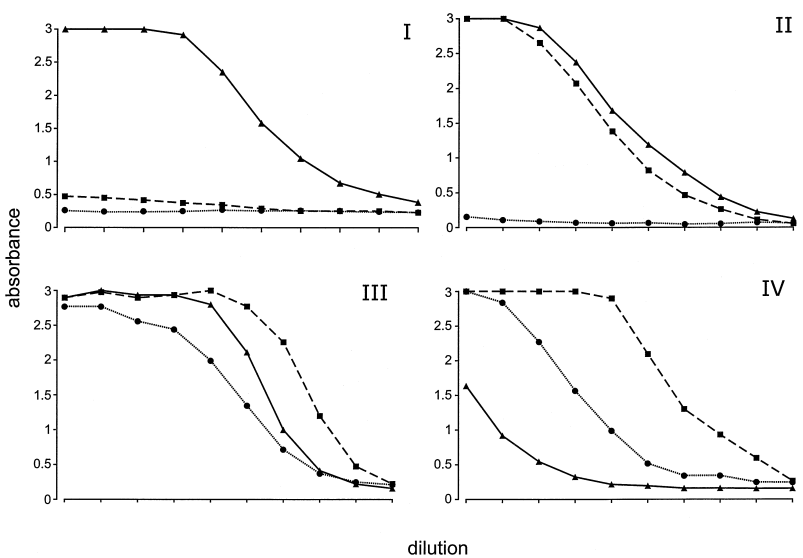

Fig. 1. Patterns of reactivity with SLPS of the various monoclonal antibodies (Mab) used in this study. — ▲ — M-SLPS, — ■ — A-SLPS, · · · • · · · Y-SLPS. Dilutions are not given, because they vary with the Mab preparations. Of the M- and C-epitope specific Mabs, two Mabs, Bm3-2 and Bm3-7, followed pattern I, four Mabs, 12G12, 2E11, Bm38 and Bm40 exhibited pattern II, one Mab, Bruce 4, showed pattern III, while the A-epitope-specific Mab 4F9 followed pattern IV.

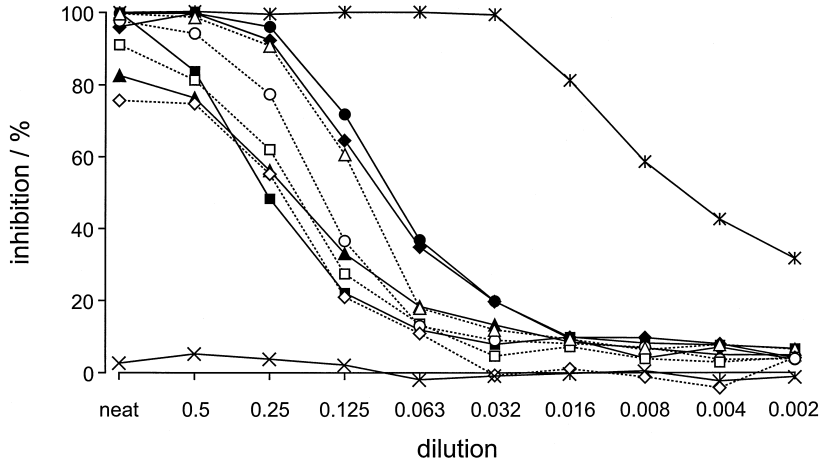

Fig. 2. Inhibition behaviour of various sera in the blocking ELISA, using M-SLPS as antigen. Closed symbols and solid lines = sera B1–B4, which are sera from *B. abortus* infected animals of comparable serological status to sera from *Y. enterocolitica* 0:9 infected heifers; open symbols and dotted lines = sera Y1–Y4, which are sera from *Y. enterocolitica* 0:9 infected heifers; — ∗ — = average of the two strong positive sera B5 and B6 from *B. abortus* infected animals; × = average of the four negative sera N1–N4. Note: similar inhibition behaviour was found for all seven M- or C-epitope-specific Mabs.

showed a pattern (type IV) with strong binding to A- and Y-SLPS. This Mab also reacted weakly with M-SLPS. It was used here to demonstrate the pattern for an A-epitope specific Mab.

3.3. Inhibition study

All Mabs except Mab 4F9, which is A-epitope specific, were used in combination with the panel of bovine sera in Table 1 in blocking ELISAs, with M-SLPS as antigen. The results for each Mab were virtually the same, following the pattern shown in Fig. 2. Strong reacting sera (B5 and B6) inhibited most strongly, while negative sera (N1–N4) showed no or little inhibition. Weak to medium strong sera B1–B4 blocked to a lower extend than the stronger sera, as expected. Unexpectedly, also the sera from *Y. enterocolitica* 0:9-infected heifers (Y1–Y4), showed inhibition behaviour virtually identical to B1–B4.

3.4. Absorption experiments

In order to identify the presence of M- and C-epitope-directed serum antibodies, the positive sera B1–B6 and the sera Y1–Y4 were incubated with Y-SLPS and the residual activity was measured in the A-, M- and Y-SLPS ELISAs. All the sera from *Y. enterocolitica* 0:9 challenged heifers and two of the serologically comparable sera from *B. abortus*-infected animals showed similar activities (Fig. 3, Y2). Antibody reactions against A-, M- and Y-SLPS were virtually equally absorbed out by Y-SLPS. Two of the

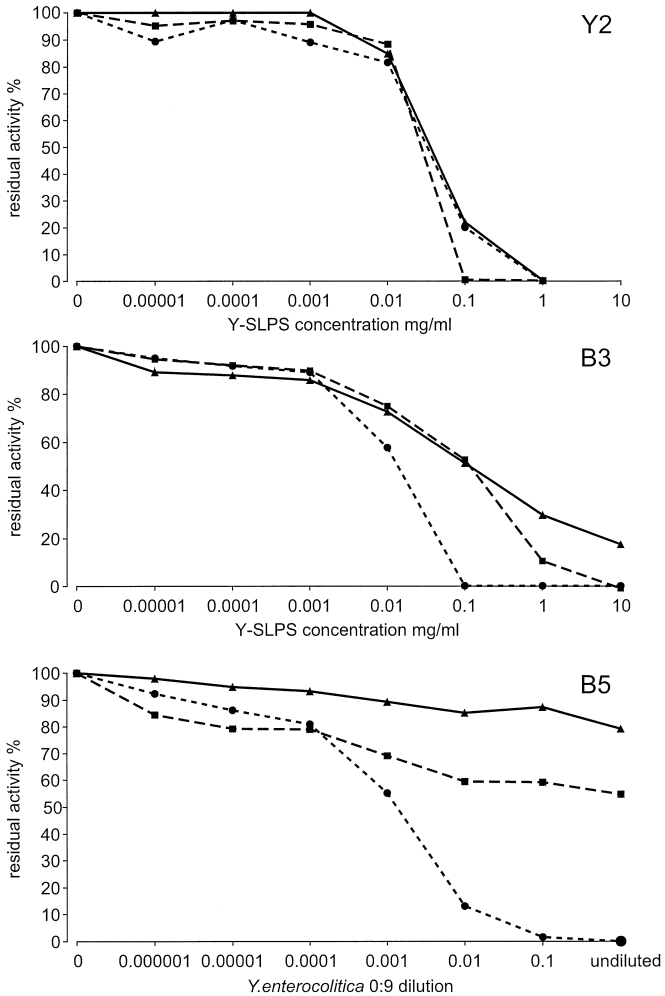

Fig. 3. Effects of absorption of various sera with Y-SLPS or *Y. enterocolitica* 0:9 on their reaction in ELISA with M-SLPS — ▲ —, A-SLPS —■— and Y-SLPS · · · • · · · as antigens. All sera from *Y. enterocolitica* 0:9 infected heifers and two sera from *B. abortus* infected animals of comparable serological status to sera from *Y. enterocolitica* 0:9 infected heifers, showed the pattern given for serum Y2. Two sera from *B. abortus* infected animals of comparable serological status to sera from *Y. enterocolitica* 0:9 infected heifers, exhibited the pattern in B3. The two strong positive sera from *B. abortus* infected animals followed the pattern shown in B5.

weaker *B. abortus* sera still exhibited residual activity against M- and A-SLPS, after complete disappearance of the Y-SLPS reactivity (Fig. 3, B3).

Because it was not possible to achieve a marked absorption of the strong *B. abortus*-positive sera B5 and B6 by using purified Y-SLPS, these sera were incubated with increasing amounts of heat-killed *Y. enterocolitica* 0:9 bacterial suspensions. While

the antibody reaction with Y-SLPS could be completely absorbed out, relatively high levels of A- and M-SLPS reactivity remained (Fig. 3, B5).

4. Discussion

This study was based on previous findings that SLPSs from *B. abortus* strains also contain, beside mostly A-epitopes, a certain number of M- and C-epitopes, which are not present in *Y. enterocolitica* 0:9 SLPS (Meikle et al., 1989; Weynants et al., 1997). If sufficient quantities of M- and C-epitope-specific serum antibodies existed during *B. abortus* infections in cattle, a sensitive test, such as a Mab-based blocking ELISA, should be able to discriminate these from false positive serum reactions caused by *Y. enterocolitica* 0:9.

Examination of the specificities of the Mabs under identical conditions confirmed the reported specificities for the Mabs. Mab 12G12, which is directed against the C-epitope (Cloeckaert et al., 1992a), was similar in its binding behaviour to the M-epitope-specific Mabs 2E11, Bm38 and Bm40. The specificity of Bruce 4 was different to the published data in that it also reacted to a certain extent with Y-SLPS. This may be due to differences in the characterisation assays used, as these were originally done with bacterial supernatant-coated plates (Bundesen et al., 1985). Nevertheless, because of their predominant reaction with M-epitopes and lack of or weak reaction with Y-SLPS, Mabs Bm3-2, Bm3-7, 2E11, BM38, BM40, Bruce 4 and 12G12 were used in blocking ELISAs.

In principle, the blocking ELISA worked well, showing strong inhibition by strong sera and less inhibition by weak sera from *B. abortus*-infected cattle. On the other hand, the results obtained with each Mab for the panel of bovine sera were surprisingly uniform, showing similar inhibition behaviour by sera from *B. abortus*- and *Y. enterocolitica* 0:9-infected cattle of comparable serological status. Similar results were obtained by others by using different and identical Mabs to this study (A. MacMillan and S. Spencer, personal communication, 1996). The inhibition by sera from *Y. enterocolitica* 0:9-infected animals was unexpected because according to previous publications (Bundesen et al., 1985; Bundle et al., 1984; Cloeckaert et al., 1992a; Greiser-Wilke and Moenning, 1987; Palmer and Douglas, 1989) M- and C-epitopes are not present in Y-SLPS and therefore serum antibodies against them should not exist in these sera. The best explanation for this observation is the binding of anti-A- and anti C/Y-epitope serum antibodies to the M-SLPS molecule. The M-SLPS used here, which is high in M-epitopes, consists of a homopolymer of unbranched pentasaccharide repeating units of four 1,2-linked and one 1,3-linked 4-amino-4,6-dideoxy-α-D-manno-pyranosyl residues (Bundle et al., 1987). The four 1,2-linked saccharides represent an A- or C/Y-epitope. Binding of anti-A- and C/Y-epitope serum antibodies, which are the majority of antibodies in sera from *B. abortus* and *Y. enterocolitica* 0:9 infected animals, possibly alter the M-epitope conformation and thus reduce the binding of M-specific Mabs in the ELISA. Furthermore, the bound A- and C/Y-specific serum antibodies may also sterically hinder the binding of the M- and C-specific Mabs.

Despite being derived from only a limited number of sera, the results of the absorbing out experiments indicate that M- and C-epitope-specific serum antibodies are present in sera from *B. abortus* infected cattle. Such antibodies seem to be present in strong sera and in half of the sera of comparable serological status to those from *Y. enterocolitica* 0:9-infected animals. Only testing of larger numbers of sera will show if absorbing out will be useful for discrimatory diagnosis. From the present results one can expect that it will be difficult in case of weak or medium strong sera.

Options to develop a specific brucellosis test are becoming increasingly limited. The continued search for Mabs or phage-display-technology-derived reagents, specific for *Brucella* SLPS epitopes which are not altered by the binding of A-epitope-specific serum antibodies, may not be productive. The most promising approach may be the improvement of the antigen-specific IFN-γ assay by using more specific *Brucella* protein antigens as, for example, those described recently by Bercovich et al. (1996) and by Denoel et al. (1997).

Acknowledgements

We wish to thank Dr. David R. Bundle for supplying us with the monoclonal antibodies Bm3-2 and Bm3-7. We are grateful for the excellent technical assistance of Mary Penrose, Gail Ross and Robyn Stark.

References

Ahvonen, P., Jansson, E., Aho, K., 1969. Marked cross-agglutination between *Brucellae* and a subtype of *Yersinia enterolitica*. Acta Pathol. Microbiol. Scand. 75, 291–295.

Alton, G.G., Jones, L.M., Angus, R.D., Verger, J.M. (Eds.), 1988. Techniques for the brucellosis laboratory. INRA, Paris.

Baldi, P.C., Guillermo, H., Giambartolomei, G.H., Goldbaum, F.A., Abdón, L.F., Velikovsky, C.A., Kittelberger, R., Fossati, C.A., 1996. Humoral immune response against LPS and cytoplasmic proteins of *Brucella* in cattle vaccinated with *Brucella abortus* S19 or experimentally infected with *Yersinia enterocolitica* 0:9. Clin. Diagn. Lab. Immunol. 3, 472–476.

Benet, J.J., Massard, C., Garin-Bastuji, B., Moutou, F., Dufour, B., Schaeffer, C., Cotton, T., 1991. Réactions sérologiques atypiques dans le dépistage de la brucellose bovine: Enquête épidémiologique dans les départements concernés. Epidémiol. Santé Anim. 19, 97–130.

Bercovich, Z., Ter Laak, E.A., 1990. An evaluation of the DTH test for diagnosing brucellosis in individual cattle: a field study. Vet. Microbiol. 22, 241–248.

Bercovich, Z., Lagendijk, W., Bokhout, B.A., 1989. Evaluation of a delayed hypersensitivity test for the diagnosis of *Brucella* infection in cattle. Vet. Immunol. Immunopathol. 21, 213–218.

Bercovich, Z., Dekker, T., Eger, A., Haagsma, J., 1996. A comparison of the potency of several *Brucella* allergens used to detect brucellosis in cattle. Vet. Res. Commun. 20, 141–151.

Bundesen, P.G., Wyatt, D.M., Cottis, L.E., Blake, A.S., Massingham, D.A., Fletcher, W.A., Street, G., Welch, J.S., Rylatt, D.B., 1985. Monoclonal antibodies directed against *Brucella abortus* cell surface antigen. Vet. Immunol. Immunopathol. 8, 245–260.

Bundle, D.R., Gidney, M.A.J., Perry, M.B., Duncan, J.R., Cherowonogrodzky, J.W., 1984. Serological confirmation of *Brucella abortus* and *Yersinia enterocolitica* 0:9 *O*-antigens by monoclonal antibodies. Infect. Immun. 46, 389–393.

Bundle, D.R., Cherowonogrodzky, J.W., Perry, M.B., 1987. The structure of the lipopolysaccharide *O*-chain (M antigen) and polysaccharide B produced by *Brucella melitensis* 16M. FEBS Lett. 216, 261–264.

Bundle, D.R., Cherowonogrodzky, J.W., Gidney, M.A.J., Meikle, P.J., Perry, M.B., Peters, T., 1989. Definition of *Brucella* A and M epitopes by monoclonal typing reagents and synthetic oligosaccharides. Infect. Immunol. 57, 2829–2836.

Caroff, M., Bundle, D.R., Perry, M.B., Cherwonogrodzky, J.W., Duncan, J.R., 1984a. Antigenic *S*-type lipopolysaccharide of *Brucella abortus* 119-3. Infect. Immun. 46, 384–388.

Caroff, M., Bundle, D.R., Perry, M.B., 1984b. Structure of the *O*-chain of the phenol-phase soluble cellular lipopolysaccharide of *Yersinia enterocolytica* 0:9. Eur. J. Biochem. 139, 195–200.

Cloeckaert, A., Jaques, I., de Wergifosse, P., Dubray, G., Limet, J.N., 1992a. Protection against *Brucella melitensis* or *Brucella abortus* in mice with immunoglobulin G (IgG), IgA and IgM monoclonal antibodies specific for common epitope shared by the Brucella A and M smooth lipopolysaccharides. Infect. Immun. 60, 312–315.

Cloeckaert, A., Kerkhoffs, P., Limet, J.N., 1992b. Antibody response to *Brucella* outer membrane proteins in bovine brucellosis: Immunoblot analysis and competitive enzyme-linked immunosorbent assay using monoclonal antibodies. J. Clin. Microbiol. 30, 3147–3168.

Cloeckaert, A., Zygmunt, M.S., Dubray, G., Limet, J.N., 1993. Characterization of *O*-polysaccharide specific monoclonal antibodies derived from mice infected with the rough *Brucella melitensis* strain B115. J. Gen. Microbiol. 139, 1551–1556.

Chukwu, C.C., 1985. Differentiation of *Brucella aborutus* and *Yersinia enterocolitica* serotype 0:9 infections: use of lymphocyte transformation test. Int. J. Zoon. 12, 126–135.

Corbel, M.J., 1985. Recent advances in the study of *Brucella* antigens and their serological cross-reactions. Vet. Bull. 55, 927–942.

Corbel, M.J., Cullen, G.A., 1970. Differentiation of the serological response to *Yersinia enterocolitica* and *Brucella abortus* in cattle. J. Hyg. Camb. 68, 519–531.

Denoel, P.A., Vo, T.K.-O., Tibor, A., Weynants, V.E., Trunde, J.-M., Dubray, G., Limet, J.N., Letesson, J.-J., 1997. Characterization, occurrence and molecular cloning of a 39-kilodalton *Brucella abortus* cytoplasmic protein immunodominant in cattle. Infect. Immun. 65, 495–502.

Douglas, J.T., Palmer, D.A., 1988. Use of monoclonal antibodies to identify the distribution of A and M epitopes on smooth *Brucella* species. J. Clin. Microbiol. 26, 1353–1356.

Garin, B., 1993. Le dépistage de la brucellose des ruminants et ses difficulté. Point Vet. 25, 115–124.

Garin-Bastuji, B., Bowden, R.A., Dubray, G., Limet, J.N., 1990. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting analysis of smooth-lipopolysaccharide heterogeneity among *Brucella* biovars related to A and M specificities. J. Clin. Microbiol. 28, 2169–2174.

Goldbaum, F.A., Leoni, J., Wallach, J.C., Fossati, C.A., 1993. Characterization of an 18-kilodalton *Brucella* cytoplasmic protein which appears to be a serological marker of active infection of both human and bovine brucellosis. J. Clin. Microbiol. 31, 2141–2145.

Greiser-Wilke, I., Moenning, V., 1987. Monoclonal antibodies and characterization of epitopes of smooth *Brucella* lipopolysaccharides. Ann. Inst. Pasteur/Microbiol. 138, 549–560.

Hilbink, F., Fenwick, S.G., Thompson, E.J., Kittelberger, R., Penrose, M., Ross, G.P., 1995. Non-specific seroreactions against *Brucella abortus* in ruminants in New Zealand and the presence of *Yersinia enterocolitica* 0:9. N.Z. Vet. J. 45, 175–178.

Kittelberger, R., Hilbink, F., Hansen, M.F., Penrose, M., de Lisle, G.W., Letesson, J.J., Garin-Bastuji, B., Searson, J., Fossati, C.A., Cloeckaert, A., Schurig, G., 1995a. Serological cross-reactivity between *Brucella abortus* and *Yersinia enterocolitica* 0:9: I. Immunoblot analysis of the antibody response to *Brucella* protein antigens in bovine Brucellosis. Vet. Microbiol. 47, 257–270.

Kittelberger, R., Hilbink, F., Hansen, M.F., Ross, G.P., Joyce, M.A., Fenwick, S., Heesemann, J., Wolf-Watz, H., Nielsen, K., 1995b. Serological cross-reactivity between *Brucella abortus* and *Yersinia enterocolitica* 0:9: II. The use of *Yersinia* outer proteins for the specific detection of *Yersinia entereocolitica* infections in ruminants. Vet. Microbiol. 47, 271–280.

Kittelberger, R., Reichel, M.P., Joyce, M.A., Staak, C., 1997. Serological crossreactivity between *Brucella abortus* and *Yersinia enterocolitica* 0:9: III. Specificity of the in vitro antigen-specific gamma interferon test for bovine brucellosis diagnosis in experimentally *Yersinia enterocolitica* 0:9-infected cattle. Vet. Microbiol. 57, 361–371.

Leong, D., Diaz, R., Milner, K., Rudbach, J., Wilson, J.B., 1970. Some structural and biological properties of *Brucella* endotoxin. Infect. Immun. 1, 174–182.

Limet, J.N., Vo, T.K.-O., Saegerman, C., de Waele, L., Tibor, A., Cloeckaert, A., de Wergifosse, P., Trunde, J.-M., Zygmunt, M., Letesson, J.-J., Dubray, G., 1992. Identification et clonage des antigènes de diagnostic de la brucellose: perspectives d'application. In: Plommet, M. (Ed.), Prevention of Brucellosis in Mediterranean Countries. Pudoc Scient. Publ., Wageningen, NL, pp. 252–264.

Limet, J.N., Cloeckaert, A., Bezard, G., Van Broeck, J., Dubray, G., 1993. Antibody response to the 89-kDa outer membrane protein of *Brucella* in bovine brucellosis. J. Med. Microbiol. 39, 403–407.

MacDiarmid, S.C., 1994. Bovine brucellosis eradication in New Zealand. Surveillance 21, 18–21.

MacDiarmid, S.C., Hellstrom, J.S., 1987. An intradermal test for the diagnosis of brucellosis in extensively managed cattle herds. Prev. Vet. Med. 4, 361–369.

MacMillan, A., 1990. Conventional serological tests. In: Nielsen, K., Duncan, J.R. (Eds.), Animal Brucellosis. CRC Press, Boca Raton, FL, USA, pp. 153–197.

Meikle, P.J., Perry, M.B., Cherwonogrodzky, J.W., Bundle, D.R., 1989. Fine structure of A and M antigens from *Brucella* biovars, Vol. 57, pp. 2820–2828.

Mittal, K.R., Tizard, I.R., 1979. A simple technique to differentiate between animals infected with *Yersinia enterocolitica* IX and those infected with *Brucella abortus*. Res. Vet. Sci. 26, 248–250.

Nielsen, K., Cherwonogrodzky, J.W., Duncan, J.R., Bundle, D.R., 1989. Enzyme-linked immunosorbent assay for the differentiation of the antibody response of cattle naturally infected with *Brucella abortus* or vaccinated with strain 19. Am. J. Vet. Res. 50, 5–9.

Palmer, D.A., Douglas, J.T., 1989. Analysis of *Brucella* lipopolysaccharide with specific and cross-reacting monoclonal antibodies. J. Clin. Microbiol. 27, 2331–2337.

Saegerman, C., Weynants, V., Vo, T.K., Dewaele, L., Tibor, A., Denoel, Ph., Godfroid, J., Michel, P., Saman, E., Letesson, J.J., Limet, J., 1994. Evaluation de l'activité protectrice de la fraction de paroi de *Brucella* insoluble dans le SDS et identification d'antigènes de *Brucella* utilisables por le diagnostic. In: AUPELF-UREF (Ed.), Biotechnologies du Diagnostic et de la Prévention de Maladies Animales. John Libbey Eurotext, Paris, pp. 221–233.

Weynants, V., Godfroid, J., Limbourg, B., Saegerman, C., Letesson, J., 1995. Specific bovine brucellosis diagnosis based on in vitro antigen-specific gamma interferon production. J. Clin. Microbiol. 33, 706–712.

Weynants, V., Tibor, A., Denoel, P.A., Saegerman, C., Godfroid, J., Thiange, P., Letesson, J.J., 1996. Infection of cattle with *Yersinia enterocolitica* 0:9 a cause of false positive serological reactions in bovine brucellosis diagnostic tests. Vet. Microbiol. 48, 101–112.

Weynants, V., Gilson, D., Cloeckaert, A., Tibor, A., Denoel, P.A., Godfroid, F., Limet, J.N., Letesson, J.-J., 1997. Characterization of smooth lipopolysaccharides and O polysaccharides of *Brucella* species by competition binding assays with monoclonal antibodies. Infect. Immun. 65, 1939–1943.