Protection conferred on mice by monoclonal antibodies directed against outer-membrane-protein antigens of *Brucella*

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Summary. Twenty-six monoclonal antibodies (MAbs) directed against seven brucella outer-membrane proteins (OMPs) of 10, 16·5, 19, 25–27, 31–34, 36–38 and 89 Kda were screened for passive protection of mice; three MAbs (directed against 16·5, 25–27 and 36–38 Kda) reduced significantly the initial colonisation of the spleen measured 7 days after challenge. Although significant, the reduction in numbers of *Brucella* organisms in the spleen was low compared with that conferred by MAbs against lipopolysaccharide of smooth specificity (S-LPS). The three most protective MAbs belonged to two isotypes (IgG1 and IgG2a) and were specific for three different OMPs. No relationship between protection and binding of MAbs to the challenge S strain or its rough mutant was observed in the mouse model. The humoral protection depended mainly on antibodies directed against S-LPS, although some MAbs against OMPs had weak protective activity.

Introduction

Brucellae are facultative intracellular parasites that cause brucellosis in animals and man. Protective immunity has been most extensively studied in a mouse model in which protection was mediated by T lymphocytes^{1,2} and polyclonal antibodies of which the different specificities were not identified.^{2–4} Opsonisation of *Brucella* organisms, either *in vitro* before injection⁵ or *in vivo* during the post-challenge bacteraemic phase,^{2,6–10} confers on mice an immune protection that may represent a large part of the vaccinal immunity.^{2,4} Whereas immune sera raised against the lipopolysaccharide extracted from smooth-cell colonies (S-LPS) and against the outer-membrane protein (OMP)-linked peptidoglycan (PG) fractions were very efficient in protection,² these sera contained too many antibody specificities to identify precisely the antigens involved (J. N. Limet, M. Plommet and G. Dubray, unpublished observations). For this reason, we used an indirect approach involving passive transfer of monoclonal antibodies (MAbs) to identify the antigens that elicit antibody-mediated protective immunity. In common with others, we demonstrated that MAbs directed against the polysaccharide O-chain of S-LPS can confer protection on mice.^{8,11,12}

Brucella S-LPS bears major A and M specificities determined with monospecific sera and defining biovars of *Brucella* spp.¹³ MAbs of A or M specificity are able to protect mice passively in relation to the A or M S-LPS specificity of the challenge strain.¹⁴ Other antigens may also be involved; *in-vitro* studies have indicated that at least two different types of antibody may help macrophages to ingest and digest *Brucella* organisms.¹⁵ In addition to S-LPS, major OMPs of the PG fraction may be putative candidates for humoral protective antigens because highly protective polyclonal anti-PG immune sera contain antibodies against these proteins.

We have now examined 26 MAbs directed against seven protein components of the outer membrane for protective ability in the mouse model.

Materials and methods

Bacterial strains

Challenge strains. *B. abortus* strain 544 (S) and (R) variants were obtained from the Station de Pathologie de la Reproduction (Nouzilly, Tours). Purity and phase were checked by standard procedures.¹³

Whole bacterial S and R cells for binding studies. Cell cultures were grown at 37°C for 48 h on slopes of Trypticase Soy Agar (TSA); BioMérieux, Marcy-

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l'Etoile, France) supplemented with yeast extract (Difco) 0.1% w/v. The bacteria were killed by addition of 20 µl of peracetic acid (Aldrich-Chimie, Strasbourg, France) 5% v/v to each 1-ml volume of 10^{10} cells/ml, followed by incubation overnight at room temperature. Before use, killing efficiency was checked by spreading 0.2-ml samples of each cell suspension, after three washes in sterile distilled water, on TSA.

Bacterial fractions. Cell walls, and insoluble cell-wall fraction (PG fraction) obtained after boiling cell walls in sodium dodecyl sulphate (SDS) 4% solution, were extracted as described previously.¹⁶ Briefly, cells were inactivated by heating at 65°C for 1 h and broken with glass beads in a Braun MSK Homogenizer or a Dyno-Mill apparatus (W. A. Bachofen, Basel, Switzerland). Crude cell walls were recovered by centrifugation (53 000 g, 4°C, 1.5 h). Cell walls from *B. melitensis* strain B115 (R) were obtained by treating crude cell walls with Triton X-100 1% in 0.2 M NaCl, 0.01 M MgCl₂ for 30 min at 20°C, then washing them six times in distilled water before lyophilisation. PG fraction from *B. melitensis* strain H38 (R) was obtained by treating crude cell walls with SDS 4% in the Laemmli sample buffer for electrophoresis. Insoluble fractions were recovered by centrifugation, washed six times in distilled water and then lyophilised.

Cell extracts of *B. abortus* strain B115 (R) were obtained by ultrasonication. Cells were inactivated by heating at 65°C for 1 h, washed three times in NaCl 0.9% and exposed to ultrasonication for 15 min in 1 mM EDTA, 30 mM Tris, pH 8. The ultrasonicated cells were then centrifuged for 10 min at 4000 g and the supernate was recovered.

LPS fractions. S-LPS fraction of *B. abortus* strain B3 (S-LPS of A>M specificity) was prepared by the phenol-water method.¹⁷

Production of monoclonal antibodies (MAbs)

BALB/c mice were immunised by three weekly intraperitoneal (i.p.) injections of 100 µg of PG fraction or cell wall. After 3 months, mice were given i.p. booster doses of 1 mg of PG fraction or cell wall. Other BALB/c mice were infected by i.p. injection of 10^9 live cells of *B. abortus* strain 45/20 (R) or strain B3 (S). After 4 months, these mice were given i.p. booster doses of 10^9 heat-killed cells of *B. abortus* strain 45/20 (R), or strain 99 (S). Four days after the booster injection, spleen cells were harvested and fused with cells of the NSO non-secreting myeloma cell line in a ratio of 5:1. After fusion, cells were suspended in selective hypoxanthine-aminopterin-thymidine-containing medium and seeded in 96-well microtitration plates at a concentration of 5×10^4 splenocytes per well. Anti-brucella hybridomas were screened by ELISA and cloned by the limiting dilution technique. Ascitic fluids were prepared in BALB/c mice according to the method of Limet *et al.*¹¹ The anti-S-LPS MAb of A specificity was the C used previously¹¹ and identified as B66/04F09 (IgG2a isotype).

MAb isotypes were determined by direct latex agglutination immunoassay of the culture supernates. Latex particles were coated with rat monoclonal anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and polyclonal anti-mouse IgA as described previously.¹⁸

Antisera

Rabbit anti-mouse immunoglobulin antiserum (RAM) was produced by repeated intradermal injections of 100 µg of mouse IgG. Rabbits were given injections of mouse IgG and bled every 2 weeks during several months. The best bleedings were pooled.

Rabbit anti-brucella antiserum was produced by repeated intradermal injections of 10^9 killed cells of *B. abortus* strain 45/20 (R) or strain 99 (S). The immunoglobulin fraction was obtained by (NH₄)₂SO₄ precipitation.

ELISA

Ascitic fluids were assayed for antibody activity by solid phase ELISA against fresh live and peracetic acid-inactivated R bacterial cells. The MAb-binding results were similar with live or killed cells and, for safety reasons, we subsequently used only peracetic acid-killed cells. For ELISA we used as diluent phosphate-buffered saline, 0.02 M, pH 7.2, (PBS) containing Tween 20 (T) 0.05% (PBS-T).¹⁵ Bacterial cells were immobilised on microtitration plates (Greiner labortechnik-Stuttgart) by capture with rabbit anti-brucella immunoglobulins at a coating concentration of 10 µg/ml. Hybridoma supernates were serially diluted (from 1 in 10 to 1 in 590490) in glycine buffer saline-EDTA-Tween (0.17 M NaCl, 0.1 M glycine, 6 mM NaN₃, 50 mM EDTA, Tween 80 0.1%, pH 9.2). Binding to whole bacterial cells was detected with peroxidase-conjugated goat anti-mouse immunoglobulins (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted in PBS-T. Excess of reagents between the different incubations was removed by five washings with saline containing Tween 20 0.05%. For substrate solution, 52 mM ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline sulphonate]; Boehringer, Mannheim, Germany) was dissolved in water and autoclaved at 121°C for 15 min. The working substrate solution was made by adding 1 ml of ABTS stock solution to 50 ml of 0.1 M sodium citrate buffer, pH 4.2, and 1 ml of H₂O₂ (125 µl of H₂O₂ 30% in 10 ml of distilled water).

The ELISA titres were estimated as the highest dilutions that gave an increase in light absorbance at 414 nm (A₄₁₄) more than twice the mean of the corresponding blank values (without MAb but with conjugate and substrate).

Protection of mice

MAbs were screened for protection of mice as described previously,^{11,14} except that two groups of

four mice were used for each MAb, at ascitic fluid dilutions of 1 in 5 and 1 in 50. MAbs were injected subcutaneously (0.1 ml) 1 day before the standard intravenous challenge with *B. abortus* strain 544 (2×10^5 cfu). The mice were killed 7 days after challenge; individual spleen counts were made and were expressed as mean $\log_{10}$ and SD cfu per group. Two successive experiments were performed, with 12 and 16 MAbs respectively. Each experiment included two control groups, one of which received saline (negative control), the other anti-S-LPS MAb of A specificity (B66/04F09, IgG2a) as positive control. The statistical significance was determined after variance analysis by one-side comparison of means with the negative control mean according to Dunnett's procedure.¹⁹

Immunoblot examination of MAbs

Protein antigens in sonicated cell extracts of *B. melitensis* strain B115 (R) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as originally described by Laemmli,²² in a 2001 vertical

electrophoresis unit (LKB-Produkter AB, Bromma, Sweden). After electrophoresis, the proteins were transferred to nitrocellulose with a Transblot apparatus (BioLyon, Dardilly, France) operated at 12 V for 2 h. Unoccupied sites on the nitrocellulose membranes were blocked by incubation for 30 min in Tris-buffered saline (TBS) with bovine serum albumin (BSA) 3% at room temperature with agitation.

The membranes were then incubated successively at room temperature overnight with hybridoma supernates diluted 1 in 2 in Tween (Tw) TBS BSA 1%, for 1 h with RAM and finally for a further hour with peroxidase-conjugated protein A, each diluted 1 in 250 and 1 in 1000, respectively, in the same buffer. Protein A (Sigma) was conjugated with horseradish peroxidase (Sigma) by a modification of the method of Nakane and Kawaoi²⁰ as described previously.²¹ The protein: enzyme ratio was 1:2. Washings between incubation periods were with Tw-TBS. After two final washings with Tw-TBS and two washings with TBS, the blots were developed by incubation at room temperature in a solution of 4-chloro-1-naphthol

Table I. Protective activity and binding of the anti-brucella OMP MAbs to R and S *Brucella* cells (experiment 1)

OMPs (M _r) Immunogen clone no.	Isotype	Mean (SD) log ₁₀ <i>Brucella</i> cfu after treatment* with MAb at dilution		Binding of MAb to <i>B. abortus</i> strain			
				544 (R)		544 (S)	
				Titre†	Max absorbance‡	Titre†	Max absorbance‡
25–27 Kda							
Live <i>B. abortus</i> B3							
A18/13D02/F05	IgG1	6.09 (0.42)	6.14 (0.14)	196830	1.518	2430	1.078
A19/12B10/F04	IgG1	5.03 (0.26)§	5.72 (0.04)	65610	1.416	90	0.492
PG fraction from H38 (R)							
A59/01E11/D11	IgG2a	6.15 (0.29)	6.52 (0.16)	270	1.009	30	0.626
A59/05F01/C09	IgG2a	6.34 (0.14)	5.84 (0.57)	65610	1.551	2430	1.231
31–34 Kda							
PG fraction from H38 (R)							
A59/10F09/G10	IgG2a	6.25 (0.36)	6.27 (0.22)	270	0.857	30	0.619
36–38 Kda							
PG fraction from H38 (R)							
A63/11E05/D11	IgG1	6.39 (0.20)	5.84 (0.43)	270	0.457	90	0.409
A63/08D08/C07	IgG1	6.19 (0.47)	6.24 (0.20)	810	0.735	270	0.592
A63/03H02/B01	IgG1	6.24 (0.26)	6.29 (0.27)	810	0.993	90	0.780
A63/05A07/A08	IgA	6.25 (0.22)	6.12 (0.30)	810	0.570	270	0.476
A63/04D11/G01	IgG2a	5.50 (0.39)§	5.93 (0.43)	270	0.886	10	0.335
A63/13G02/C04	IgG1	6.39 (0.16)	6.05 (0.26)	810	1.294	270	0.826
89 Kda							
Cell wall from B115 (R)							
A53/10B02/A01	IgG1	6.20 (0.41)	6.45 (0.15)	270	0.590	10	0.314
S-LPS (A)							
S-LPS fraction, <i>B. abortus</i> B3							
B66/04F09 diluted 1 in 200	IgG2a	4.98 (0.14)§	...	10	0.117	810	2.001
Control: saline		6.30 (0.59)	...				
Control: ascites no anti-brucella activity				90	0.304	30	0.422

* MAbs were injected subcutaneously 1 day before challenge with 200 000 cfu of strain 544(S); mice were killed after 7 days and Brucellae from spleen were counted.

† Higher dilution that gave an A_{414} value greater than twice the blank value.

‡ Maximal A_{414} value after subtraction of blank value.

§ $p < 0.01$ in comparison with untreated control groups (one-sided Dunnett's test).

(BioRad, Richmond, USA) 0.06% w/v and 5 mM H₂O₂ in TBS. The reaction was stopped by washing with distilled water.

Results

Protection conferred on mice

In experiment 1 (table I), MAb A19/12B10/F04 (anti-25–27 Kda OMP) and MAb A63/04D11/G01 (anti-36–38 Kda OMP) produced significant reductions of spleen counts at day 7 at the 1 in 5 dilution ($d' = 0.64$, $p < 0.05$; $d' = 0.80$, $p < 0.01$). In experiment 2 (table II), MAb A19/12B10/F04 and MAb A18/13D02/F05 (anti-25–27 Kda OMP) (negative results

in experiment 1) were tested again with 14 others. MAb A19/12B10/F04 was protective at both dilutions (5.21 $p < 0.01$, 5.57 $p < 0.05$; $d' = 0.62$, $p < 0.05$; $d' = 0.78$, $p < 0.01$). MAb A66/05H01/E09 (anti-16.5 Kda OMP) was protective at the 1 in 5 dilution (5.54 $p < 0.05$) but MAb A18/13D02/F05 (anti-25–27 Kda OMP) gave most efficient protection at the 1 in 50 dilution (5.60, $P < 0.05$). The negative and positive control results were as expected.

Overall, three MABs out of 26 reduced spleen counts at day 7 in the mouse model. The three protective MABs were obtained from mice immunised with living virulent *Brucella* (S) organisms, or with cell walls and PG fractions from *B. melitensis* strains 115 (R) and H38 (R). These MABs belonged to isotypes IgG1 and IgG2a and reacted specifically with OMPs of 16.5, 25–27, and 36–38 Kda.

Table II. Protective activity and binding of the anti-brucella OMP MABs on R and S *Brucella* cells (experiment 2)

OMPs (M _r) Immunogen clone no.	Isotype	Mean (SD) Log ₁₀ <i>Brucella</i> cfu after treatment* with MAB at dilution		Binding of MAb to <i>B. abortus</i> strain			
				544 (R)		544 (S)	
				5	50	Titre†	Max absorbance
10 Kda							
Live <i>B. abortus</i> 45/20							
A68/08E07/B11	IgG2a	5.91 (0.08)	5.82 (0.34)	810	0.672	30	0.479
A68/07G11/C10	IgG2a	5.73 (0.16)	5.92 (0.38)	810	1.049	30	0.435
16.5 Kda							
Live <i>B. abortus</i> 45/20							
A68/29E03/C10	IgG2a	5.95 (0.37)	6.26 (0.16)	7290	1.320	30	0.650
A68/04G01/C06	IgG2a	5.82 (0.62)	5.79 (0.46)	810	1.270	30	0.713
Cell wall from B115 (R)							
A66/04E03/B07	IgG2b	5.82 (0.48)	5.93 (0.33)	810	0.670	2430	0.538
A66/05H01/E09	IgG2a	5.54 (0.23)	5.78 (0.20)	270	0.438	30	0.262
A66/04E05/E09	IgG2a	5.86 (0.20)	5.78 (0.35)	270	0.514	90	0.373
19 Kda							
Live <i>B. abortus</i> 45/20							
A68/25H10/A05	IgG2a	5.99 (0.26)	6.02 (0.14)	90	0.424	<10	0.018
25–27 Kda							
Live <i>B. abortus</i> B3							
A18/13D02/F05	IgG1	6.16 (0.16)	5.57 (0.46)	196830	1.518	2430	1.078
A19/12B10/F04	IgG1	5.21 (0.28)§	5.60 (0.18)	65610	1.416	90	0.492
Cell wall from B115 (R)							
A66/01C06/E01	IgG2b	5.88 (0.22)	5.94 (0.18)	7290	1.313	90	0.559
Live <i>B. abortus</i> 45/20							
A68/07D11/B03	IgG2a	5.85 (0.25)	5.93 (0.12)	196830	1.244	2430	1.370
A68/04B10/F05	IgG2a	5.84 (0.45)	5.99 (0.22)	65610	1.474	810	1.367
36–38 Kda							
PG fraction from H38 (R)							
A63/04F07/E03	IgM	5.98 (0.34)	5.98 (0.21)	65610	2.430	2430	2.023
Live <i>B. abortus</i> 45/20							
A68/15B06/C08	IgG2a	5.73 (0.34)	5.98 (0.22)	21870	0.990	90	0.627
A68/25G05/A05	IgG2a	5.94 (0.27)	5.90 (0.33)	196830	1.324	2430	1.571
S-LPS (A)							
S-LPS fraction, <i>B. abortus</i> B3							
B66/04F09 diluted 1 in 200	IgG2a	4.40 (0.24)§	...	10	0.117	810	2.001
Control: saline		6.22 (0.35)	...				
Control: ascites non anti- <i>Brucella</i>				90	0.304	30	0.422

*, †, ‡, § see footnotes to table I.

|| $p < 0.05$.

Determination of OMP specificity by Western immunoblot analysis

The specificity of the protective anti-OMP MABs was checked by Western immunoblotting with sonicated cell extracts of *B. melitensis* strain B115 (R) as antigen (figure). Multiple bands (doublets or triplets) were observed with the anti-25–27 and –36–38 Kda MABs. A single band was observed for the anti-16·5 Kda protective MAB.

Binding of MABs to the brucella cell surface

The ability of MABs to bind to the cell surface of intact cells of *B. abortus* strain 544 R and S variants was determined by ELISA (tables I and II). Most of the anti-OMP MABs showed better binding to *B. abortus* strain 544 R cells than to S cells. Most of the anti-10, -16·5 and -19 Kda MABs bound only to R cells. The major OMPs of 25–27 and 36–38 Kda were much less accessible on S cells than on R cells. No correlation was found between protective activity of the MABs and their ability to bind to the cell surface. Some MABs directed against the 25–27 Kda OMP bound more effectively to the cell surface than the

anti-25–27 Kda protective MAB but were not able to protect mice from infection.

Discussion

Polyclonal immune sera raised against the PG fraction that reacted strongly with OMPs and S-LPS in immunoblots were highly protective in the mouse model.^{2,4} Having shown that MABs directed against the LPS A or M dominant antigen conferred good protection,^{11,13} we produced 26 MABs against OMPs¹⁶ which were screened in our mouse model for protection. Three were able to induce a significant reduction of spleen counts at day 7 after challenge. Using a similar approach, Montaraz *et al.*⁸ obtained protection with O-polysaccharide (LPS)-specific MABs but no protection with two anti-porin (37–38 Kda) IgM MABs. The protection conferred by our three anti-OMP MABs even if highly significant, was quite low compared with that conferred by the S-LPS positive control MAB (B66/04F09). The low protective effect induced by anti-OMP MABs may be related to weaker opsonisation resulting from poor fixation at the cell surface, possibly because of low accessibility and avidity. One MAB showed a protective activity only at a dilution of 1 in 50; a detrimental effect following high-dose antibody administration in animals challenged with *Staphylococcus aureus* has been shown²³ which may be due to blockade of the immune system. There was no clear correlation between binding of anti-OMPs MABs to the challenge S strain and its R mutant and protection. This probably means that the binding of MABs to cells *in vitro* is only a part of the protective action of MABs *in vivo*. A complex mechanism of action of antibodies may operate during ingestion and killing of *Brucella* organisms by macrophages¹⁴ and antibody-dependent cell-mediated cytotoxicity may also be involved.²⁴

MABs protective against other gram-negative bacteria have been described.^{25–28} However, most mouse models of protection are based on lethality and comparisons are difficult. Nevertheless, Sawada *et al.* showed that MABs to OMPs were much less protective than MABs to S-LPS against infection of mice with *Pseudomonas aeruginosa*.²⁷

The role of anti-OMP antibodies in immunity to brucellosis remains uncertain. Using the MABs we have developed, it should be possible to investigate the effects of these MABs *in vivo* on the first phase of the infection, the possible synergy of two or more MABs, and the mechanisms at the cellular level of digestion and killing of intracellular *Brucella* organisms.

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Figure. Immunoblot of sonicated heat-killed cell extracts of *B. melitensis* strain B115 (R) with (1) anti-16·5 Kda MAB A66/05H01/E09, (2) anti-25–27 Kda MAB A19/12B10/F04 and (3) anti-36–38 Kda MAB A63/04D11/G01.

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