Humoral immunity in mice mediated by monoclonal antibodies against the A and M antigens of *Brucella*

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Summary. All smooth strains of *Brucella* bear two lipopolysaccharide (LPS) antigens in a ratio that defines the classification of strains in serovars, A ($A > M$), M ($M > A$) and A.M ($A = M$). Anti-LPS-A monoclonal antibodies (MAb-A) were previously shown to convey protection to mice against *B. abortus* (A) strain 544, as shown by lower spleen counts than in controls at days 7 and 21 after challenge. Anti-LPS-M monoclonal antibodies (MAb-M) were obtained and tested for M-specificity with LPS from reference strains by ELISA, by agglutination of LPS-coated latex particles, and by inhibition of this agglutination. Antigens A and M of three strains were quantified by a homologous LPS-latex and MAb agglutination inhibition assay. Protection conferred by MAb-A and MAb-M against three strains, *B. abortus* 544 (A), *B. abortus* 292 (M) and *B. melitensis* H38 (M), was tested at equivalent challenge and MAb doses: intravenous challenge was adjusted to give similar infection at day 7; MAb doses were adjusted to the same specific ELISA titre. Under these conditions, MAb-A and MAb-M conferred both early and late protection, as shown at days 7 and 21, against the strains that bore the homologous major antigen, *i.e.*, strain 544 on one hand and strains H38 and 292 on the other. In contrast, MAb directed against the minor antigen of the challenge strain conferred significant protection at day 7 only with strains 544 and H38 and no or inconsistent protection against strain 292, which expressed the lowest amount of minor antigen. Thus, early and late antibody-mediated immune mechanisms depend on amounts of surface LPS antigens accessible to specific antibodies. Therefore, to protect against the various strains of *Brucella*, an LPS-based vaccine should induce high titres of specific antibodies against both A and M antigens.

Introduction

Protective immunity against *Brucella*, demonstrated by splenic or hepatic bacterial counts in mice challenged intravenously, can be transferred from donor to recipient by either immune lymphoid cells or serum (Pavlov *et al.*, 1982; Plommet and Plommet, 1983, 1987). Whereas antigen(s) involved in cell-mediated immunity are likely protein component(s) of the SDS-I fraction (Dubray, 1987; Winter, 1987), lipopolysaccharide (LPS) antigens are involved in humoral immunity transferred by monoclonal antibodies (Montaraz *et al.*, 1986;

Limet *et al.*, 1987; Vendrell *et al.*, 1987). The *Brucella* cell in the smooth phase bears two LPS antigens, the ratio of which defines the serovars: antigen A dominant over antigen M ($A > M$) in serovar A and *vice versa* in serovar M, or serovar A,M in equivalence (Wilson and Miles, 1932; Stableforth and Jones, 1963). This classification with, originally, A thought to represent *B. abortus* and M *B. melitensis*, was later recognised to be independent of the species. Three anti-A monoclonal antibodies (MAb-A) were previously shown to confer protection against an A-dominant *B. abortus* challenge strain (Limet *et al.*, 1987). In this investigation we used MAb-A and MAb-M to examine the involvement of the major (dominant) antigen in immune protection.

Materials and methods

Bacterial strains

Three strains of *Brucella* were used for challenge and for LPS extraction: *B. abortus* 544, (ATCC 23448) biovar 1 serovar A (A > M), *B. abortus* 292 (ATCC 23451) biovar 4 serovar M (M > A), and *B. melitensis* H38 biovar 1 serovar M (M > A), a highly virulent strain used in ruminants (Renoux, 1955) and experimental infection of mice (Bosserey *et al.*, 1982, 1984). The challenge strains were harvested in phosphate-buffered saline from a 24-h culture on Blood Agar Base (Difco). The suspension was adjusted photometrically to the required concentration and viable counts (cfu) were determined retrospectively on five plates.

Two other strains, *B. melitensis* strain 16 M, which is the reference strain for biovar 1 serovar M, and a field strain of *B. abortus*, biovar 3 serovar A, were used for LPS extraction by the phenol-water method (Baker and Wilson, 1965). LPS was used to coat latex particles (Limet *et al.*, 1988).

LPS extraction

Crude LPS of proteinase K-digested whole-cell lysates (PK-LPS) was extracted from the five different strains of *Brucella* as described by Dubray and Limet (1987), with some modifications. Cells of a 48-h culture on blood agar base in Roux flasks were harvested in distilled water and inactivated by heating at 60°C for 1 h. The cells were centrifuged (12 000 *g*, 4°C, 30 min) and suspended in 0.0625 M Tris-HC buffer containing sodium dodecyl sulphate (SDS) (0.5 g wet weight of cells/10 ml) 2% w/w. Samples were heated at 100°C for 10 min and the lysates were cooled to 55°C. Proteinase K (Boehringer, Mannheim, FRG) was added (0.15 mg/ml) and the samples were incubated at 55°C for 3 h. The samples were then held overnight at 20°C and SDS 0.02 g/ml was added before heating at 100°C for 10 min. The samples were centrifuged (12 000 *g*, 20°C, 30 min) and the LPS contained in the supernate was precipitated by addition of an equal volume of isopropanol. After 30 min, the precipitate was harvested by centrifugation (12 000 *g*, 4°C, 30 min) and dissolved in 10 ml of distilled water. After a second isopropanol precipitation, the pellets were treated with RNAase and DNAase, both 0.01 mg/ml, at 37°C for 30 min, then by proteinase K 0.01 mg/ml at 55°C for 3 h and then 20°C overnight. After a third isopropanol precipitation, the pellets containing LPS were recovered in 1 ml of distilled water and lyophilised.

Monoclonal antibodies

Monoclonal antibodies (MAbs) were prepared with a non-secretory myeloma cell line (Shulman *et al.*, 1978) as described in Van Snick and Coulie (1982) and Limet *et al.* (1987) except that for anti-M, mice were immunised with three subcutaneous injections (200 µl) of the Abor-

lane (*B. melitensis* H38) vaccine (Rhône Mérieux, Lyon, France) at 10-day intervals. One or 3 months later, mice were given booster injections of 100 µg of LPS from *B. melitensis* 16 M.

Ascitic fluids were produced by intraperitoneal (i.p.) injection of 5×10^6 hybridoma cells to pristane-treated and irradiated BALB/c mice. MAbs were purified by affinity chromatography on protein A sepharose.

Immunoassays

Reagents. Glycine-buffered saline (GBS; 0.1 M glycine, 0.17 M NaCl, 6.15 mM, NaN₃, pH 9.2) was used alone or supplemented with 50 mM EDTA (GBS-EDTA) or with 50 mM EDTA and bovine serum albumin 10 mg/ml (GBS-EDTA-BSA; BSA = 98% electrophoretically pure; Calbiochem-Behring Corp., La Jolla, USA) or with Tween 20 0.05% v/v (GBS-Tween).

Enzyme-linked immunosorbent assay of anti-LPS antibodies. Microtitration plates (Greiner, Labortechnik, Stuttgart, FRG) were coated by overnight incubation at 37°C with LPS 1 µg/ml in GBS 20% in water. Plates were either stored at 4°C or washed, dried and sealed in plastic bags containing a dessicant capsule. Anti-LPS antibodies were serially diluted in GBS-Tween and were incubated in plates at room temperature for 1 h, then washed five times with 0.15 M NaCl containing Tween 0.1% v/v. Horseradish peroxidase-labelled goat anti-mouse IgG diluted in GBS-Tween containing fetal calf serum 2% was added. The plates were re-incubated at room temperature for a further 1 h. Peroxidase activity remaining after five washes was revealed by incubation for 20 min with 0.2 mM H₂O₂ and 4 mM O-phenylenediamine in 0.024 M citrate, 0.051 M phosphate buffer, pH 5. The reaction was stopped with 1 M sulphuric acid and the absorbance was read at 492 and 620 nm.

Latex agglutination immunoassay. The immunoassay for the antigen and antibody titrations was performed in an automated immunoassay analyser, in which the degree of latex agglutination was determined by counting the number of non-agglutinated particles (Masson *et al.*, 1981). The analyser, controlled by a Hewlett Packard 85 computer, dilutes, mixes, incubates and records the number of non-agglutinated particles from which the analyte concentration is interpolated from a dose-response curve (figs 1 and 2).

Latex coating. Latex particles were coated with LPS (A or M; Limet *et al.*, 1988) by incubation of 50 µl of latex suspension (K109 Rhône-Poulenc, Courbevoie, France) 10% w/v with LPS 100 µg in 0.4 ml of GBS 20% in water. The mixture was sonicated (Branson, Danbury, England) for 30 s and, after 10 min, stabilised by adding 200 µg of human serum albumin (Behringwerke, Marburg/Lahn, FRG) in GBS 20%, and incubated at room temperature for 20 min. The LPS-coated latex suspension was centrifuged at 10 000 *g* for 10 min and the pellet was resuspended in 1 ml of SDS 1% w/v, sonicated again, and incubated at 37°C for 1 h. It was then twice washed with 1 ml of GBS 20%, once with 1 ml of GBS-EDTA-BSA and resuspended in 1 ml of this buffer. LPS-coated latex

Fig. 1. Specificity of MAb-A 04F9 (a) and MAb-M 16C10 (b) assessed by inhibition of LPS (A and M respectively)-coated latex particles agglutination by PK-LPS from *B. abortus* strain 544 (A > M), (○) or *B. melitensis* strain H38 (M > A), (■).

Fig. 2. Quantitation of A and M antigens on challenge strains by agglutination inhibition with protein K-treated bacteria of the three strains. Agglutination of LPS-A latex by MAb-A (open symbols) and LPS-M latex by MAb-M (closed); *B. abortus* strain 544 (○, ●), *B. abortus* strain 292 (◇, ◆), *B. melitensis* strain H38 (□, ■).

was stored in small volumes at -20°C for up to 2 years without loss of agglutinability. When thawed, LPS-coated latex was sonicated for 15 s and diluted 50-fold in GBS-EDTA-BSA supplemented with rheumatoid factor 0.1% v/v. Rheumatoid factor serum from a patient with rheumatoid arthritis was used to enhance the weak agglutinating activity of IgG antibodies. This suspension could also be stored lyophilised for more than 9 months without loss of activity.

Agglutination inhibition. Inhibition of agglutination of LPS-coated latex particles by MAb was performed as follows: (1) 30 μl of samples containing different amounts

of PK-LPS were vortex-mixed for 30 min with 30 μl of MAb diluted in GBS-EDTA-BSA; (2) 30 μl of a suspension of latex particles 0.1% w/v containing rheumatoid factor was added and incubated for 12 min; (3) free particles were then assayed (this number is proportional to LPS concentration).

Determination of conferred immunity

Outbred CD-1, 6–7-week-old female mice born at the station were distributed randomly in groups of five, 1 week before transfer. MAbs (0.1 or 0.2 ml) diluted in phosphate buffered saline, or buffer alone in control groups, were injected intravenously (i.v.) or subcutaneously (s.c.) 1 day before challenge. MAb 2E-11 induced an immediate lethal shock in some mice when injected i.v.; no difference between the i.v. and s.c. routes was observed in a comparative assay. The *Brucella* challenge (0.2 ml) was injected i.v. at appropriate doses. The mice were killed 7 or 21 days later for *Brucella* spleen counts, as described by Bosseray *et al.* (1984). Results were given as the mean $\log_{10}$ cfu/experimental group and either the SD per group or the residual deviation of the experiment.

The statistical significance between the control and the MAb-recipient mice was calculated by variance analysis, using the common variance of the experiment, unless otherwise stated, in non-orthogonal individual comparisons with one degree of freedom for the F-test (Steel and Torrie, 1960).

Results

Characteristics of the MAbs

Two MAb-Ms were used, the characteristics of which are given in table I, in comparison with MAb-A 04F9 used previously (Limet *et al.*, 1987).

Table I. Characteristics of monoclonal antibodies

MAb	Specificity	Isotype*	A/M ratio†		
			ELISA‡	LPS latex agglutination‡	Agglutination inhibition§
04F9	A	IgG _{2a}	0.0003	0.0003	<0.01
2E11	M	IgG ₃	388	388	>100
16C10	M	IgG ₃	10.7	13	333

*Determined in ELISA with LPS-coated plates and specific antisera from Bio-Rad, Richmond, CA, USA.

†LPS-A from *B. abortus* biovar 3 (A > M) and LPS-M from *B. melitensis* 16M biovar 1 (M > A).

‡ELISA or agglutination MAb titres ratio LPS-A/LPS-M.

§Ratio of LPS-A/LPS-M concentrations that gave 50% inhibition of agglutination in specific MAb-LPS-coated latex system, i.e., LPS-A with MAb 04F9 and LPS-M with MAbs 2E11 or 16C10.

The highly specified MAb-M 2E11 was used in protection experiments. The MAb-M 16C10 which presented a very similar specificity, at least when tested in agglutination inhibition assay, was used to quantify the amounts of M epitopes in the PK-LPS extract of challenge strains because it presents a higher affinity than MAb-M 2E11. MAbs 04F9 and 16C10 are probably largely specific to antigen A and M respectively, as shown by the inhibition observed with the PK-LPS of two different reference strains (fig. 1); if the reaction of MAb 16C10 with LPS A is considered to be a cross-reaction, the latter is, in all cases, <1%.

Quantitation of A and M antigens on challenge strains

Measurement of A and M antigens was performed by agglutination inhibition assay; different amounts of PK-LPS from the challenge bacteria were used to inhibit the agglutination of LPS-A- or

LPS-M-coated latex particles by MAb-A or MAb-M (fig. 2). As the inhibition is proportional to the amount of LPS added, the quantity of A and M antigen in the LPS of each strain can be expressed either by the amount that gives a stated inhibition, or by the ratio of the amounts that give the same inhibition with one MAb (table II). It has been observed previously (data not shown) that the inhibition by whole bacterial cell preparations was similar to that by PK-LPS, but high concentrations of bacterial cells interfere with particle counting. Therefore, this limits the range to a one-log scale.

By using proteinase K-treated cells, it was possible to show that, on the three strains, the minor antigen was only a small fraction of the major one: 0.325% for antigen M on strain 544 compared with strain 292 (100%) and 0.045% for antigen A on strain 292 compared with strain 544 (100%). Interestingly, between the two M > A strains, the respective amounts of major and minor antigens differed in opposite directions; there was

Table II. Comparative quantitation by agglutination inhibition of LPS A and M antigens of challenge bacteria

MAb (specificity)	LPS on the latex	Amounts of proteinase K-treated bacteria (mg/ml) giving the same inhibition* with strains		
		H38 (M)†	292 (M)†	544 (A)†
16C10 (M)	LPS M	56 (46.4)‡	26 (100)	8000 (0.325)
04F9 (A)	LPS A	3600 (0.125)	10 000 (0.045)	4.5 (100)

*50% for 16C10 and 12% for 04F9.

†Major antigen according to Wilson and Miles (1932).

‡Relative amounts (%) of A and M antigens on the strains, with strain 544 and strain 292 for A and M reference (100%) respectively, are shown in parentheses.

2.16 times more M antigen and 2.77 times less A antigen on strain 292 than on strain H38.

Determination of equivalent challenge doses

For a strain of mice, the time course of splenic infection depends on the virulence of the challenge strain and the immunity of the host. Thus, different immune effectors should be compared at equivalence of virulence of the challenge strains. For this, the challenge doses have to be adjusted to give a similar splenic infection in control mice at least 7 days after challenge.

As shown in fig. 3: (1) the two strains of *B. abortus* gave similar responses—splenic infections were 6.2 and 5.7 mean log₁₀ cfu at day 7 and 4.7 and 4.8 mean log₁₀ cfu at day 21 for strains 544 and 292, respectively, with challenge doses of 1.5×10^5 and 1.3×10^5 cfu; (2), in contrast, to reach similar results at day 7, *B. melitensis* strain H38 was injected at a lower dose, 2×10^4 cfu. With this highly virulent strain, the infection remained at a high level at day 21.

Protection conferred by MAb-A and MAb-M against A- and M-dominant challenge strains

Comparison of immune protection conferred by MAb-A and MAb-M should be performed on an equivalent activity basis. For this, the smallest dose of MAb-A still active against *B. abortus* strain 544 (A > M) was first determined. A dose of 4 µg still conferred protection, without significant differ-

ences at higher doses at day 7 and day 21 (not shown). The equivalent weight of MAb-M, in ELISA, to 4 µg of MAb-A was estimated from table I to be 537 µg. Mice were given either MAb-M (537 µg) or MAb-A (4 µg), then challenged with the three strains.

Immunity conferred by MAb-A against *B. abortus* 544 was highly significant at days 7 and 21 ($p < 0.001$ and $p = 0.01$ respectively), whereas protection conferred by MAb-M was significant at day 7 only ($p < 0.001$) and not at 21 days ($p > 0.05$) (fig. 4). Immunity against *B. abortus* strain 292 conferred by MAb-M was significant at days 7 and 21 ($p < 0.001$ and $p < 0.05$), whereas MAb-A conferred a small protection at day 7 only ($p < 0.05$).

Immunity against *B. melitensis* strain H38 was highly significant with MAb-M at days 7 and 21 ($p < 0.001$ and $p = 0.001$ respectively; fig. 4). With this strain, SD at day 21 was larger than at day 7; therefore, comparisons were calculated separately. Immunity conferred by MAb-A was again significant at day 7 ($p < 0.001$), but no longer significant at day 21 ($p > 0.05$). A 2.5 times higher dose of MAb-A did not increase protection (not shown).

Discussion

Because *Brucella* are facultative intracellular bacteria, T-cell dependent immunity has been considered, since Mackaness (1964), to be the main mechanism of immune protection. However, it has often been shown that humoral effectors participate to a large extent in the immune mechanisms at the

Fig. 3. Spleen counts at 7(a) and 21(b) days after challenge doses of three strains: *B. abortus* strain 544 (●), *B. abortus* strain 292 (□), *B. melitensis* strain H38 (■); mean ± SD; n = 5.

Fig. 4. Protection conferred by MAb-A or MAb-M (2E11) at equivalent dose against three strains injected at equivalent challenge doses; *B. abortus* strains 544 and 292, 2.0×10^5 ; *B. melitensis* strain H38, 1.1×10^4 cfu; ■, MAb-A, 4 μ g; ○, MAb-M, 537 μ g; ●, control; bar = residual standard deviation; n = 5.

cellular level and in protection against a virulent challenge, as established by the spleen-count model in mice (Plommet and Plommet, 1983). The nature of the antibodies involved has not been clearly demonstrated. Using rabbit monospecific, absorbed A and M sera, Sulitzeanu *et al.* (1955) suggested a highly specific protection in a mouse model with i.p. injection. From in-vitro experiments with macrophages, Ralston and Elberg (1971) assumed that at least two antibodies against cell-wall antigens were involved. Having developed a mouse model (Bossery *et al.*, 1982, 1984), we compared the protection conferred by mouse immune sera raised against LPS and protein cell wall (PG) antigens; both antibodies were shown to be efficient (Plommet and Plommet, 1983), but analysis of our immune sera by absorption, electrophoresis and immunoblotting did not allow clear identification of the antigen(s) involved. We have shown that three MAb-As (IgG1, IgG2a, IgG3) were protective, in the model, against the *B. abortus* 544 (A) strain (Limet *et al.*, 1987). Similar results were obtained by Montaraz *et al.* (1986) with two MAb-As (IgG2a, IgG3) against *B. abortus* 2308 (A) challenge strain. Vendrell *et al.* (1987) compared five MAbs belonging to three isotypes but of unknown specificity and titre against a *B. suis* (A)

bacteria, exhibiting different amounts of A and M antigens.

Specificity of our MAbs was established by ELISA, agglutination of LPS coated latex particles, and inhibition of this agglutination by LPS extracted from reference strains of each type. Correlation with the A and M label, according to the classical definition of Wilson and Miles (1932), was also confirmed by additional agglutination-inhibition assays with other reference strains (not shown) that exhibited the characteristic A and M profiles in SDS-polyacrylamide gel electrophoresis (Dubray and Limet, 1987).

Using two MAbs directed against the A or the M antigen, we established the role of each antigen in humoral immune protection at equivalence of antibody titres and of the virulence of the challenge strain in the outcome of infection. Efficiency of immune mechanisms during the first two phases of brucella infection depends mainly on the major LPS antigen of the challenge strain. During the first phase, when trapping of circulating bacteria inside the liver and lymph nodes is the main mechanism (Plommet and Plommet, 1983), antibodies against both major and minor antigens may be efficient. Trapping inside the reticuloendothelial system may require only a small amount of surface LPS antigen.

major LPS-antigen. Even the highly virulent *B. melitensis* strain H38, that is able to multiply quickly during the first phase and to resist killing during the second phase in control mice, was shown to be highly susceptible to MAb-M-mediated protection in both phases.

Two main proteins of the cell membrane of 25–27 and 36–38 Kda considered to be mainly involved in cell-mediated immunity on the basis of T-cell transfer experiments (Plommet and Plommet, 1983) may also act through antibody-dependent immunity (Dubray 1987; Winter 1987). Two IgM MAbs directed against the outer-membrane protein of 36–38 Kda (porin) have already been shown to be inefficient (Montaraz *et al.*, 1986), but other MAbs of different isotypes and of higher affinity or other specificity need to be used in passive immunisation experiments before a definitive conclusion can be drawn.

Nevertheless, these results obtained with a more virulent strain confirm the importance of the LPS as protective antigen in the mouse model and define more precisely the role of the dominant antigen. Therefore, to be protective against all *Brucella* strains, a vaccine based on the LPS should induce antibodies against both A and M epitopes. LPS derived from strains bearing A and M antigens in similar amount (Stableforth and Jones, 1963) should probably be preferred to A > M or M > A strains in which the minor antigen is very weakly represented (<1%). This hypothesis remains to be checked.

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