Protection conferred on mice by combinations of monoclonal antibodies directed against outer-membrane proteins or smooth lipopolysaccharide of *Brucella*

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Summary. The effect of monoclonal antibodies (MAbs) injected alone or in combination on brucella splenic infection in CD-1 mice was tested 7 and 21 days after a challenge with virulent *Brucella abortus* 544. Passive immunisation of mice with anti-25–27-kDa MAb alone, or mixed with protective anti-16·5 and anti-36–38-kDa MAbs, or with MAbs of the same specificity which were previously demonstrated to have no activity on CD-1 mice, produced a significant reduction of spleen counts of *B. abortus* ($p < 0·01$). Other combinations of MAbs did not reduce splenic infection in comparison with the untreated control group. BALB/c mice were used to test the possible interference of the immune response of CD-1 mice against MAbs that were produced in BALB/c mice. No reduction of splenic infection was shown with anti-25–27- or -36–38-kDa MAbs, whereas anti-lipopolysaccharide (LPS) MAb which was produced in CBA mice was effective. Combination of anti-protein MAbs with the anti-LPS MAb produced only the effect of the anti-LPS MAb at 7 and 21 days after challenge.

Introduction

Brucella spp. are gram-negative facultative intracellular bacteria that cause acute and chronic infection in man and animals. Protective immunity to brucellosis, as studied in the mouse model, can be transferred from donor to recipient mice by either immune serum or T-cells.^{1–3} T-cell dependent immunity has been considered, since the work of Mackaness,⁴ to be the main mechanism of immune protection. However, passive transfer of monoclonal antibodies (MAbs) directed against outer-membrane proteins (OMPs)⁵ or smooth lipopolysaccharide (S-LPS) of *Brucella*^{6–9} confers some immunity to brucella infection in mice. We demonstrated that three MAbs directed against the 16·5-, 25–27- and 36–38-kDa OMPs reduced significantly the colonisation of the spleen at 7 days after challenge.⁵ Immunity conferred by anti-S-LPS MAbs of A or M specificity is in relation to the A or M S-LPS specificity expressed by the challenge strain. Nevertheless, the protection conferred by MAbs directed against OMPs, although statistically significant, was quite low compared with that conferred by the S-LPS MAb. For this reason, we investigated the synergic action of mixtures of MAbs against several epitopes of one OMP, or against epitopes on several OMPs on infection in CD-1 mice, and the effect of MAbs against OMPs and S-LPS on infection in BALB/c mice. As

most anti-OMP MAbs were obtained from BALB/c mice, the strain of mice could influence the protective response and this possibility was examined.

Materials and methods

Bacterial strain

B. abortus strain 544, used as challenge strain, was from the culture collection held at INRA-Nouzilly. Purity and phase were checked by standard procedures.¹⁰ Cloned smooth colonies were transferred on to Blood Agar Base (BAB; Difco) slants for 24 h. *Brucella* cells were then harvested in a sterile buffered saline solution (BSS; NaCl 8·5 g, K₂HPO₄ 2 g, KH₂PO₄ 1 g, distilled water 1000 ml, pH 6·85) and adjusted photometrically at 600 nm to the required concentration (optical density = 0·165 for 10⁹ cfu/ml for 1-cm light path) and injected into mice as indicated for each experiment.

Monoclonal antibodies (MAbs)

MAbs were produced as described previously.⁵ They are listed in tables I and II. MAbs were used at a final dilution of 1 in 5, except for the anti-S-LPS MAb of A specificity (B66/04F09) which was diluted 1 in 200 (minimal effective dose determined by Limet *et al.*⁷).

Protection of mice

Mice were acclimatised for 1 week before use, and

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randomly allocated to experimental groups 5 days before immunisation. Each experiment included two controls—the anti-LPS of A specificity MAb (B66/04F09) as positive control and BSS as negative control.

Experiment A. The synergic activity of MAbs directed against OMPs was tested on groups of 12 outbred CD-1, 5-week-old female mice (Charles River, Elbeuf, France). The combinations of MAbs used are given in table I. MAbs found previously to decrease brucella colonisation of spleen⁵ were injected alone: A66/05H01/E09 (anti-16·5-kDa OMP), A19/12B10/F04 (anti-25–27-kDa OMP) and A63/04D11/G01 (anti-36–38-kDa OMP). MAbs (0·1 ml final volume) diluted in BSS, and BSS alone (control), were injected subcutaneously 1 day before challenge. Mice were challenged intravenously with 0·2 ml of a suspension containing $5·3 \log_{10}$ cfu of *B. abortus* 544.

Experiment B. Groups of 18 5-week-old female BALB/c mice (Iffa-Credo, L'Arbresle, France) were used to test the effect of the combination of MAbs directed against OMP and the MAb directed against the S-LPS (A) on the protection against *B. abortus* 544. Mice were given $4·4 \log_{10}$ cfu of this strain by intravenous injection 1 day after immunisation with MAbs.

Six mice from each group were killed at stated times (experiment A, days 7 and 21; experiment B, days 7, 21 and 49) and their spleens were removed. The number

of *B. abortus* organisms in each spleen were counted by grinding, diluting and spreading on BAB.^{11,12} The number of cfu of *B. abortus*/spleen (x) was expressed as $y = \log x$. This last transformation normalises the distribution of individual counts, required for variance analysis.¹³ Mean and SD of transformed values were then calculated. The results were evaluated by analysis of variance of all effects and interactions.¹⁴

Results

Reduction of splenic infection in CD-1 mice by mixtures of anti-OMP MAbs

The results of these experiments are shown in table I. Anti-25–27-kDa MAb (A19/12B10/F04) injected alone decreased splenic infection ($p < 0·05$) in day 7 after challenge with virulent *B. abortus*, whereas anti-16·5-kDa MAb (A66/05H01/E09) reduced splenic infection on day 21 after challenge only ($p < 0·05$). Anti-36–38-kDa MAb (A63/04D11/G01) did not decrease splenic colonisation at either stage. The injection of these three MAbs in combination was very efficient at protecting mice, as well as the combination of MAb anti-25–27-kDa with other MAbs of the same specificity ($p < 0·01$). However, the difference between reduction of spleen counts by these combinations and by the anti-25–27-kDa MAb alone was not significant.

Table I. Protective activity of anti-OMP MAbs against *B. abortus* 544 (S)

Clone	MAb specificity	Protection*	Isotype	Mean (SD) $\log_{10}$ <i>Brucella</i> cfu at days post-challenge	
				7	21
...	Control (saline)	—	...	6·07 (0·21)	4·60 (0·29)
A66/05H01/E09	16·5 kDa	+	IgG2a	5·88 (0·41)	4·10 (0·41)†
A19/12B10/F04	25–27 kDa	+	IgG1	5·52 (0·25)†	4·28 (0·43)
A63/04D11/G01	36–38 kDa	+	IgG2a	6·13 (0·44)	4·48 (0·28)
A66/05H01/E09	16·5 kDa	+	IgG2a	5·37 (0·05)‡	4·23 (0·28)
A19/12B10/F04	25–27 kDa	+	IgG1		
A63/04D11/G01	36–38 kDa	+	IgG2a		
A19/12B10/F04	25–27 kDa	+	IgG1	5·35 (0·24)‡	4·48 (0·41)
A66/01C06/E01		—	IgG2b		
A68/04B10/F05		—	IgG2a		
A68/07D11/B03		—	IgG2a		
A18/13D02/F05		—	IgG1		
A66/01C06/E01	25–27 kDa	—	IgG2b	5·83 (0·28)	4·61 (0·43)
A68/04B10/F05		—	IgG2a		
A68/07D11/B03		—	IgG2a		
A18/13D02/F05		—	IgG1		
A66/04E03/B07		—	IgG2b		
A66/04E05/E09	16·5 kDa	—	IgG2a	5·75 (0·33)	4·42 (0·34)
A68/04G01/C06		—	IgG2a		
A68/29E03/C10		—	IgG2a		
A68/07G11/C10	10 kDa	—	IgG2a	6·09 (0·30)	4·18 (0·47)†
A68/08E07/B11		—	IgG2a		
A63/04F07/E03		—	IgM		
A68/25G05/A05	36–38 kDa	—	IgG2a	5·13 (0·18)‡	3·90 (0·41)‡
A68/25H10/A05	19 kDa	—	IgG2a		
B66/04F09	S-LPS (A)	+	IgG2a		

* Protection observed by Cloeckaert *et al.*⁵

† $p < 0·05$, ‡ $p < 0·01$, in comparisons with untreated control group according to analysis of variance.

Table II. Protective activity of anti-OMP MAbs alone or in combination with the anti-LPS MAb against splenic infection with *B. abortus* 544

Treatment (clone)	Isotype	Mean (SD) log ₁₀ <i>Brucella</i> cfu, at days post-challenge		
		7	21	49
Control (BSS)	...	6.12 (0.06)	7.78 (0.03)	6.54 (0.23)
Anti-LPS (B66/04FO9)	IgG2a	5.14 (0.09)	6.17 (0.41)	6.83 (0.23)
Anti-25-kDa OMP (1) (A19/12B10/F04)	IgG1	5.97 (0.09)	7.60 (0.04)	6.11 (0.15)
Anti-25-kDa OMP (2) (A18/13D02/F05)	IgG1	6.24 (0.10)	7.74 (0.08)	6.28 (0.18)
Anti-36-kDa OMP (1) (A63/04D11/G01)	IgG2a	6.29 (0.08)	7.67 (0.06)	6.33 (0.07)
Anti-36-kDa OMP (2) (A68/25G05A05)	IgG2a	6.11 (0.06)	7.67 (0.07)	6.53 (0.35)
Anti-25-27-kDa OMP (1) + anti LPS	IgG1 + IgG2a	5.19 (0.04)	5.51 (0.39)	6.30 (0.42)
Anti-25-kDa OMP (2) + anti LPS	IgG1 + IgG2a	5.34 (0.08)	5.49 (0.49)	6.78 (0.18)
Anti-36-38-kDa OMP (1) + anti LPS	IgG2a + IgG2a	4.97 (0.05)	6.42 (0.08)	7.19 (0.23)
Anti-36-kDa OMP (2) + anti LPS	IgG2a + IgG2a	5.05 (0.04)	5.64 (0.27)	6.76 (0.09)

(1), (2), different MAbs of the same specificity.

Other combinations of MAbs failed to decrease splenic colonisation of CD-1 mice, except the combination of anti-10-, anti-19- and anti-36-38-kDa on day 21 after challenge. Control (BSS and anti-LPS MAb) results were as expected; anti-LPS MAb conferred protection at both times ($p < 0.01$), BSS had no effect.

Reduction of splenic infection in BALB/c mice by anti-OMP and by anti-OMP and anti-LPS MAbs

Determination of equivalent challenge dose. For a *Brucella* strain, the time course of splenic infection depends on its virulence and on the resistance of the mice. To compare the efficacy of various MAbs, the challenge dose was adjusted to give a similar splenic infection in BALB/c mice as in CD-1 mice, at least 7 days after challenge. The level of splenic infection in BALB/c mice 7 days after injection of various doses of

B. abortus 544 was determined. At that time, mean *B. abortus* log₁₀ cfu of control CD-1 mice was nearly 6, with a challenge dose of 5.3 log₁₀ cfu. This count was produced in BALB/c mice by 4.4 log₁₀ cfu of the same strain.

Reduction of splenic infection in BALB/c mice by mixtures of anti-OMP and anti-LPS MAbs

Individual anti-OMP MAbs injected alone, irrespective of specificity and isotype, did not produce any reduction of splenic infection (tables II and III). Controls (BSS and anti-LPS MAb) were as expected: the anti-LPS MAb showed the usual level of protection (5.14) observed in CD-1 mice, 7 days after challenge; 21 days after challenge, the level of infection in the control group (BSS) increased to 7.78 log₁₀ cfu (table II). Nevertheless, anti-LPS MAb was still effective ($p < 0.01$; table III) at this time whereas on day 49, it was not. The combination of anti-OMP MAb with the anti-LPS MAb produced significant reduction of splenic counts ($p < 0.01$) on days 7 and 21 after challenge (table III). On day 49 after challenge, no reduction of splenic infection was found, whatever the treatment administered to mice (table III).

Discussion

In previous studies, we have shown that MAbs directed against LPS A or M antigens^{6,7} and three MAbs directed against OMPs⁵ are able to significantly reduce splenic infection in CD-1 mice resulting from a challenge with *B. abortus* strain 544. We have extended this observation in two ways. Firstly, we tested whether synergic action of mixtures of anti-OMP MAbs of the same specificity or of several specificities could improve reduction of spleen colonisation in CD-1 mice. Secondly, we tested whether anti-OMP MAbs would improve the reduction of spleen colonisation when

Table III. Statistical analysis of experiment B

Treatment comparison*	Error: MS†	F
On day 7		
Control vs anti-LPS	0.025	115.6‡
Control vs anti-OMP	0.025	0.202
Control vs mixtures	0.025	203.6‡
Interactions		
anti-LPS × anti-25-27-kDa OMP	0.025	0.12
anti-LPS × anti-36-38-kDa OMP	0.025	3.50
On day 21		
Control vs anti-LPS	0.382	20.3‡
Control vs anti-OMP	0.382	0.017
Control vs mixtures	0.382	51.05‡
Interactions		
anti-LPS × anti-25-27-kDa OMP	0.382	2.60
anti-LPS × anti-36-38-kDa OMP	0.382	0.008
On day 49		
Control vs anti-LPS	0.293	0.84
Control vs anti-OMP	0.293	0.77
Control vs mixtures	0.293	0.65

* d.f. = 1 for treatment comparison.

† Mean square, d.f. = 50.

‡ $p < 0.01$.

injected with the anti-LPS MAb in BALB/c mice. Immunising with anti-25–27-kDa MAb (A19/12B10/F04) alone⁵ or with MAbs of other specificities decreased spleen counts significantly. The combination of anti-25–27-kDa MAb (A19/12B10/F04) with anti-16.5 MAb (A66/05H01/E09) and anti-36–38-kDa MAb (A63/04G11/G01) or with the other MAbs of 25–27-kDa specificity, reduced splenic infection significantly when compared with unprotected control mice ($p < 0.01$). These results showed that a combination of MAbs did not inhibit the activity of anti-25–27-kDa MAb (A19/12B10/F04), even if the other MAbs in combination were not effective themselves; protection was not potentiated either. Synergic activity of MAbs was shown by Svennerholm *et al.*¹⁵

A combination of MAbs directed against several epitopes of colonisation factor antigens enhanced protection against enterotoxigenic *Escherichia coli* experimental infection. However, the effect observed was primarily an antitoxic effect, whereas in experimental brucellosis, the effect was a decrease of splenic infection. Two MAbs—A66/05H01/E09 (anti-16.5-kDa OMP) and A63/04D11/G01 (anti-36–38-kDa OMP)—that previously reduced splenic colonisation in CD-1 mice⁵ were no longer protective. This could be ascribed to the statistical distribution and to individual receptivity of the animals.

To test the interference of an eventual immune response of CD-1 mice against MAbs produced in BALB/c, the second experiment was performed with the BALB/c mice as subjects. This did not improve the reduction of splenic infection in mice treated with anti-OMP MAb, whereas the anti-LPS MAb reduced splenic colonisation as effectively as in CD-1 mice. Thus, mixing anti-OMP MAbs with the anti-LPS MAb gave a result similar to the effect of the anti-LPS

MAb alone. This also means that the binding of the anti-LPS MAb was not affected by anti-OMP MAbs.

The failure to observe a protective effect in BALB/c mice 49 days after challenge with anti-LPS MAb is in accord with the results of Phillips *et al.*⁸ who observed an increase in splenic colonisation 8 weeks after immunisation with MAbs directed against the O-polysaccharide of the LPS. The increased splenic colonisation between 7 and 21 days, and the lower challenge dose needed to achieve the same degree of colonisation as for CD-1 mice indicated that BALB/c mice were more sensitive to brucella infection. This could also explain the observation that anti-OMP MAbs did not decrease splenic colonisation in BALB/c mice. Passively administered anti-LPS MAbs could decrease brucella infection in mice, as observed on days 7 and 21 after challenge with anti-LPS MAb, by the opsonisation of bacteria during the first hours of the infection. At that time, the possible anti-immunoglobulin response against MAbs is not yet developed and the possible influence of the strain would be minimised. Short-term protection produced by passive immunisation with MAbs in the mouse model is only one aspect of the immune mechanisms which operate against *Brucella* spp. Cellular immunity probably interacts with humoral immunity. OMPs probably play a major role in cellular immunity and a minor role in humoral immunity. Their precise contribution will be evaluated by active immunisation with recombinant proteins.

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