IMMUNOLOGICAL RESPONSE TO INFECTION

Outer-membrane protein- and rough lipopolysaccharide-specific monoclonal antibodies protect mice against *Brucella ovis*

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Summary. *Brucella ovis*, a naturally virulent rough *Brucella* species, is the aetiological agent of ram epididymitis. The identification of protective antigens is necessary to obtain a safe, specific subcellular vaccine. Monoclonal antibodies (MAbs) directed at both brucella outer-membrane proteins (OMPs) and rough lipopolysaccharide (R-LPS) in a mouse protection test were used to identify potential targets for humoral immunity. Mixtures of MAbs directed at the 16·5-, 25–27-, 31–34- and 36–38-kDa OMPs conferred significant protection 7 days after challenge with reference strain *B. ovis* 63/290 compared with controls receiving either saline or an anti-brucella O-polysaccharide MAb. Furthermore, an anti-R-LPS MAb tested alone conferred protection at a level comparable with that obtained with the mixture of anti-OMP MAbs. The combination of protective OMP MAbs with the anti-R-LPS MAb was also strongly protective. One combination of OMP MAbs, which bound intensely to *B. ovis* *in vitro*, was ineffective. These results indicate that *B. ovis* OMPs and R-LPS are targets for protective antibodies and that they can be regarded as candidates for ram epididymitis subcellular vaccines.

Introduction

Brucella ovis is the aetiological agent of ram epididymitis.^{1,2} This condition causes infertility in males and may result in abortion and stillbirths in ewes and weak newborn lambs. Thus, *B. ovis* infections are of economic concern in sheep breeding. Control measures include elimination of rams found positive in serological tests and vaccination when prevalence is high. Although no *B. ovis* vaccines have been marketed so far, *B. melitensis* Rev1 vaccine, currently used to control ovine and goat brucellosis caused by *B. melitensis*, has been shown also to confer protection against *B. ovis*.³ Nevertheless, in those areas in which *B. melitensis* is not present, the use of the Rev 1 vaccine can complicate serology¹ mainly because of the induction of antibodies to O-polysaccharide (OPS) of smooth strains.

The outer membrane of *B. ovis* has been studied by several groups searching for antigens useful for diag-

nosis^{4–10} and for protection.^{11,12} A hot-saline extract (HS) from *B. ovis*^{1,6} has been found to protect mice and rams in experimental conditions. This preparation contains outer-membrane proteins (OMPs) and the rough lipopolysaccharide (R-LPS).^{8,13} The former are involved in the protective activity observed.^{11,12} It has also been reported that HS-induced protection is mainly antibody-mediated.¹² However, the immunological identity of the antigens involved in protection against *B. ovis* remains to be elucidated.

This study provides preliminary evidence of the protective activity of monoclonal antibodies (MAbs) specific for both brucella OMP and R-LPS epitopes against *B. ovis* in mice.

Materials and methods

Bacteria and cultures

The reference strain *B. ovis* 63/290, from the INRA Nouzilly *Brucella* culture collection, was grown for 24 h on Tryptose-soy Agar (Difco) supplemented with horse serum 5% in an atmosphere with CO₂ 10% as recommended.¹⁴

Received 7 April 1995; accepted 25 April 1995.

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Table. Binding of OMP- and R-LPS-specific MAbs on to *B. ovis* cell surface

Mab denomination	Specificity* (OMP kDa)	Ig isotype	ELISA	
			Max absorbance	Log titre†
A76/08C03/G03 (a)†	16.5	G2a	1.79	5.77
A68/29E03/C10 (a')	16.5	G2a	0.82	2.43
A59/01E11/D11 (b)	25–27	G2a	0.65	1.95
A59/05F01/C09 (b')	25–27	G2a	2.16	5.29
A59/10F09/G10 (c)	31–34	G2a	2.10	5.77
A68/15B06/C08 (d)	36–38	G2a	2.34	3.39
A63/04D11/G01 (d')	36–38	G2a	1.08	2.43
A68/03F03/D05 (e)	R-LPS	G2b	3.00	5.77
04/F09 (f)	OPS	G2a	0.70	1.95

ELISA values were obtained by testing individual MAbs against whole-cell *B. ovis* 63/290.

* Denomination of OMPs are given after their apparent molecular mass, i.e. 16.5 being OMP of 16.5 kDa. OPS, O-polysaccharide moiety of smooth lipopolysaccharide; R-LPS, rough lipopolysaccharide.

† Single letter denominations were used to simplify description of MAb mixtures in the figure.

‡ Titre was established as the last MAb dilution giving an *E* (414 nm) $\geq$ twice as high as the blanks.

Monoclonal antibodies

The MAbs used were as described previously.^{15–21} Their denominations and characteristics are given in the table. For ELISA determinations, the cells were washed in sterile phosphate-buffered saline, pH 7.4, heat-inactivated (65°C, 60 min) and cooled, and adjusted to an optical density of 1.0. This suspension was distributed (100 μ l/well) in 96-well flat-bottomed microtitration plates and the assay was conducted in the conditions described previously.^{15,16} Titres were

determined by interpolating in the slope at an absorbance value twice as high as the blanks.

Mouse protection test

Protection conferred by MAbs to mice was screened as described previously¹⁶ with some modifications. Briefly, MAbs were prepared, individually or in mixtures, in five-fold dilutions of ascitic fluid in PBS and then filter-sterilised. Female BALB/c mice (6–7 weeks-old, four mice/group) were inoculated subcutaneously (0.1 ml of each MAb dilution) the day before infection with 2×10^6 cfu of *B. ovis* 63/290. Mice were killed 7 days after challenge for individual spleen counts, which were expressed in mean log cfu (SD)/group. The experiment included two control groups, one inoculated with saline, and the other with anti-OPS MAb (MAb 04F09), OPS being absent from *B. ovis*. Statistical significance of differences was determined after variance analysis by comparison of group means to the negative control mean by Dunnett's procedure, with the InStat® (Abacus, Cal) software.

Results

Binding of MAbs on to *B. ovis* 63/290 as determined by ELISA is shown in the table. For each OMP (except for the 31–34-kDa OMP) the two MAbs tested showed different binding activity in terms of both maximal absorbance value and titre. Thus, the order of binding activity in ELISA was A76/08C03/G03 > A68/29E03/C10 (16.5-kDa OMP); A59/05F01/C09 > A59/01E11/D11 (25–27-kDa OMP) and A68/15B06/C08 > A63/04D11/G01 (36–38-kDa OMP).

Figure. Evaluation of the protective activity against *B. ovis* of mixtures of OMP- and R-LPS-specific MAbs in BALB/c mice. †For brevity, MAbs are given single letters; the original denominations and characteristics are given in the table. ‡Bars and error bars represent the mean and SD, respectively, of the number of cfu of *B. ovis* 63/290 in spleens after log transformation determined 7 days after an intraperitoneal challenge with 2×10^6 bacteria.

The highest binding activities were observed with anti-31–34-kDa OMP and the anti-R-LPS MABs.

A high number of viable *Brucella* organisms were found at necropsy in the spleens of non-immunised mice challenge-infected with *B. ovis* 63/290 (figure). Furthermore, mice that had received MAB 04/F09, specific for the OPS of *Brucella* spp, showed no significant differences from the non-immunised group. Significant protection was not observed with the mixture of MABs A76/08C03/G03 (16.5-kDa OMP), A59/05F01/C09 (25–27-kDa OMP) and A68/15B06/C08 (36–38 kDa). Addition of MAB A59/10F09/G10 (31–34-kDa OMP) to these MABs resulted in a significant protection level ($p < 0.05$). MABs A68/29E03/C10, A59/01E11/D11 and A63/04D11/G01 together with MAB A59/10F09/G10 gave the highest level of protection (3.94 log units, $p < 0.01$). Anti-R-LPS MAB A68/03F03/D05 injected alone was very effective, inducing a protection level comparable with that obtained with the last mentioned combination of anti-OMP MABs. When MAB A68/03F03/D05 was tested in association with the most protective mixture of anti-OMP MABs (MABs A68/29E03/C10, A59/01E11/D11, A59/10F09/G10 and A63/04D11/G01), the level of protection (4.44 log units) was even greater than that produced by the administration of the anti-OMP MABs and anti-R-LPS MAB separately.

Discussion

The results indicate that both OMP- and R-LPS-specific MABs can confer protection against *B. ovis* infection in BALB/c mice. In this study, passive protection experiments with MABs were used, an approach already used in the study of protective immunity against infections by naturally smooth *B. abortus* and *B. melitensis* strains.^{16, 18–23}

Anti-OMP MABs were selected on the basis of their binding on to whole *B. ovis* 63/290 cells in ELISA. For in-vivo assessment of protective capacity, MABs were tested in mixtures that included one MAB for each of the OMPs, grouping them according to their binding activity in ELISA. However, MAB binding was not correlated with the protective activity observed. Indeed, the mixture of MABs A76/08C03/G03 (16.5-kDa OMP), A59/05F01/C09 (25–27-kDa OMP) and A68/15B06/C08 (36–38 kDa), which bound better than MABs A68/29E03/C10, A59/01E11/D11 and A63/04D11/G01 specific for the same OMPs, provided lower protection than the latter when tested together with MAB A59/10F09/G10 (31–34 kDa). The mixture of MABs A76/08C03/G03 (16.5-kDa OMP), A59/05F01/C09 (25–27-kDa OMP) and A68/15B06/C08 (36–38-kDa OMP) gave no protection. Although the MAB specific for the 31–34-kDa OMP was not tested individually, the increase in protection observed when it was included in other MAB mixtures suggested that this MAB would be active by itself. Thus, the differences in levels of

protection observed could be attributed either to intrinsic activity of the MABs or, alternatively, to detrimental interactions between MABs, such as a blocking effect.

Differences in activity between mixtures of anti-OMP MABs would indicate that caution should be taken in the choice of OMPs constituting a vaccine against *B. ovis*. To date, conservation of antigenicity among *B. ovis* strains has been demonstrated only by testing immunoblots with polyclonal immune sera.¹³ If particular epitopes of OMPs behave differently, as the results of the present study would suggest, then the possibility of antigenic variability should also be considered. In previous studies, MABs to brucella OMPs were ineffective^{16, 17, 22} or slightly protective^{16, 17} against experimental infections with smooth brucella. In contrast, MABs directed to the A, M or common epitopes of the OPS were protective.^{18–20, 22, 23} However in *B. ovis*, a naturally rough *Brucella* species, OPS is lacking; thus, OMP epitopes are readily accessible to MABs (R. A. Bowden, unpublished observations) which may in part explain the differences in protective activity observed.

The protection obtained with anti-R-LPS MAB A68/03F03/D05 suggests that R-LPS may be an interesting target for protective immunity to the naturally rough *B. ovis*. However, we have already shown that this MAB was ineffective in controlling smooth *B. abortus* infection in mice.²¹ This difference could be explained as being due to a lower accessibility of the MAB to R-LPS epitopes in smooth *Brucella* strains.²¹ On the other hand, *B. ovis* possesses well-exposed R-LPS epitopes, easily bound by MABs. Others have claimed that R-LPS would not be an interesting candidate for a *B. ovis* vaccine because of its relatively poor immunogenicity in mice.¹² Nevertheless, it has been shown previously in active immunisation studies that HS extract from which R-LPS had been extensively depleted resulted in lower protective activity in rams than the crude, untreated HS,¹¹ suggesting a protective role for R-LPS. The results presented here with a single anti-R-LPS MAB indicate that induction of antibodies to R-LPS would be valuable to control *B. ovis* infection. Therefore, the immunogenicity of *B. ovis* R-LPS should be studied further.

In mice, antibodies raised by immunisation with *B. ovis*¹² or rough *B. abortus* RB51²⁴ have been shown to contribute to a greater extent than T cells to the control of *B. ovis* infection. This study confirmed the protective activity of antibodies against *B. ovis* and identified OMPs and R-LPS as targets for protective MABs. Experiments are under way to examine further whether single OMPs and other R-LPS MABs could confer protection and to identify individually the OMPs involved in protection.

We thank J. M. Verger and M. Grayon for supplying the *B. ovis* reference strain 63/290. R.A.B. thanks INRA and Facultad de Ciencias Veterinarias, UNICEN, Tandil, Argentina for support. A.C. received a post-doctoral fellowship from the CEC.

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