Demonstration of peptidoglycan-associated *Brucella* outer-membrane proteins by use of monoclonal antibodies

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(Received 13 December 1991; revised 16 March 1992; accepted 18 March 1992)

A monoclonal antibody (3D6) was produced which reacted only with *Brucella* sonicated cell extracts that had been lysozyme-treated after sonication. The monoclonal antibody (mAb) reacted with the three major outer-membrane proteins (OMPs) of *B. melitensis* B115 in Western blots. A large number of reactive bands ranging from 12 to 43 kDa were present in lysozyme-treated *Escherichia coli* and *Yersinia enterocolitica* sonicated cell extracts. In a latex agglutination inhibition immunoassay, mAb 3D6 showed better reactivity with purified peptidoglycan (PG) of *B. melitensis* B115 than with that of *Escherichia coli*. This mAb was also used in immunogold electron microscopy with whole *Brucella* cells and sections. No binding was observed on whole cells and immunogold labelling in sections was observed close to the outer membrane, in the periplasmic space and in the cytoplasm. These findings indicate that mAb 3D6 is specific for PG subunits. Immunoblot analysis of *B. melitensis* B115 rough sonicated cell extracts after SDS-PAGE, with or without lysozyme treatment, was performed using mAbs specific for *Brucella* OMPs of molecular masses of 10, 16.5, 19, 25–27, 31–34, 36–38 and 89 kDa, for PG and for rough lipopolysaccharide (R-LPS) and smooth lipopolysaccharide (S-LPS). mAbs specific for the 25–27, 31–34 and 36–38 kDa OMPs reacted with three to six bands. All of them except the band of lowest molecular mass reacted with the PG-specific mAb and not with R-LPS- and S-LPS-specific mAbs. Therefore we propose that variation in the apparent molecular mass of the major OMPs (25–27, 31–34, and 36–38 kDa) is due to varying numbers of PG subunit residues rather than attached R-LPS or S-LPS molecules. Our results suggest a very strong, possibly covalent, interaction of the major OMPs (25–27, 31–34 and 36–38 kDa) with PG. There is no evidence of association of the minor OMPs (10, 16.5, 19 and 89 kDa) with PG, since mAbs specific for these proteins reacted with single bands that were not apparent when reacted with the anti-PG mAb. Furthermore, lysozyme treatment did not affect the electrophoretic mobility of these proteins.

Introduction

The *Brucella* cell envelope is a three-layered structure in which an inner or cytoplasmic membrane, a periplasmic space, and an outer membrane can be differentiated (Dubray & Plommet, 1976). *Brucella* cell walls consist of a peptidoglycan (PG) layer strongly associated with the outer membrane. The outer membrane contains LPS, proteins and phospholipids. The major *Brucella abortus*

outer-membrane proteins (OMPs) have molecular masses of 36–38 kDa and 25–27 kDa (Dubray, 1987; Dubray & Bézard, 1980; Dubray & Charriaut, 1983). They are also called group 2 porin proteins and group 3 proteins (Douglas *et al.*, 1984; Moriyon & Berman, 1983; Santos *et al.*, 1984; Verstraete *et al.*, 1982, 1984). These two major OMPs are also found in the SDS-insoluble (SDS-I) cell wall fraction (Dubray, 1987; Dubray & Bézard, 1980; Dubray & Charriaut, 1983). Cell wall and SDS-I fractions of *Brucella melitensis* contain another major protein of molecular mass 31–34 kDa, which is minor in *B. abortus* strains (Dubray, 1981). A lipoprotein covalently linked to PG has also been described as a major OMP (Gomez-Miguel & Moriyon, 1986; Gomez-

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Abbreviations: mAb, monoclonal antibody; OMP, outer-membrane protein; PG, peptidoglycan; RAM, rabbit anti-mouse immunoglobulin serum; R-LPS, S-LPS, rough, smooth LPS.

Miguel *et al.*, 1987). Other OMPs identified so far are minor species with molecular masses of 10, 16.5, 19 and 89 kDa (Cloeckaert *et al.*, 1990). The 89 kDa OMP is probably a protein of group 1 with a molecular mass of 88–94 kDa described by others (Verstraete *et al.*, 1982, 1984). All these OMPs are surface-exposed as demonstrated by immunoelectron microscopy (Cloeckaert *et al.*, 1990; Gomez-Miguel *et al.*, 1987).

In a previous paper (Cloeckaert *et al.*, 1990) we showed that monoclonal antibodies (mAbs) to the 25–27, 31–34 and 36–38 kDa OMPs reacted with multiple bands in immunoblots after SDS-PAGE of lysozyme-treated sonicated *Brucella* cell extracts. Our purpose in the present study was to determine the origin of the heterogeneity of the major OMPs, whether associated with PG, R-LPS or S-LPS as suggested by Douglas *et al.* (1984). These hypotheses were investigated by analysing sonicated cell extracts with or without lysozyme treatment by use of mAbs specific for OMPs, PG, R-LPS and S-LPS.

Methods

Bacterial strains. The rough *B. melitensis* strain B115 was obtained from the Institut National de la Recherche Agronomique, Laboratoire de Pathologie Infectieuse et Immunologie, Nouzilly, Tours, France. *Escherichia coli* O:157 was obtained from the Institut National de Recherches Vétérinaires, Brussels, Belgium. *Yersinia enterocolitica* O:9 was obtained from the Microbiology Department of the Catholic University of Louvain (G. Wauters).

Sonicated cell extracts. Cell extracts of *B. melitensis* B115 were obtained by ultrasonication. Cells were inactivated by heat at 65 °C for 1 h, washed three times in 0.9% NaCl, ultrasonicated for 15 min in 1 mM-EDTA, 30 mM-Tris pH 8 and, when appropriate, digested with lysozyme. The ultrasonicated cells were then centrifuged for 10 min at 4000 g, and the supernatant recovered. Sonicated cell extracts of *E. coli* O:157 and *Y. enterocolitica* O:9 were prepared in the same way and digested with lysozyme.

Preparation of PG from *E. coli* and *Brucella*. PG was prepared by classical techniques with some modifications (Rosenthal *et al.*, 1983; Swim *et al.*, 1983; Amano & Williams, 1983). *E. coli* and *B. melitensis* B115 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 (at 53000 g, 4 °C for 1.5 h). SDS-I fraction was obtained by treating crude cell walls for 1 h in boiling 4% (w/v) SDS solution in Laemmli sample buffer. The SDS-insoluble fraction was recovered by centrifugation, washed six times in distilled water and then lyophilized. The SDS-I fraction was treated with NaOH to hydrolyse components that were noncovalently and covalently bound to the PG (Amano & Williams, 1983). SDS-I fraction (100 mg) was suspended in 4 ml 1 M-NaOH and incubated overnight at 20 °C. After neutralization with HCl and centrifugation, the pellet was resuspended in 4% SDS solution in Laemmli sample buffer and incubated for 5 min at 100 °C. After centrifugation, the pellet was washed four times in Laemmli buffer without SDS and resuspended in 4 ml distilled water. To 3 ml of this suspension, 75 µl 12 M-HCl and 2 mg pepsin were added. After one night of pepsin treatment at 20 °C, the suspension was

neutralized by NaOH (750 µl of a 1.2 M solution). After centrifugation the pellet was treated in 4% SDS solution in Laemmli sample buffer for 5 min at 100 °C, washed four times in Laemmli sample buffer without SDS and finally treated with lysozyme as described by Dubray & Charriat (1983).

Purification of the 25–27 kDa OMP. The proteins of the *B. melitensis* B115 SDS-I fraction were separated by SDS-PAGE. The 25–27 kDa OMP was electroeluted from the gel in a Biotrap system (CERA-LABO, Paris, France) using 25 mM-Tris, 192 mM-glycine, 0.1% SDS buffer (pH 8.3). Protein concentration was determined by the BCA protein assay (Pierce).

mAbs. The mAbs against OMPs, S-LPS and R-LPS were obtained as described previously (Cloeckaert *et al.*, 1990). The mAbs used were the anti-10 kDa mAb A68/07G11/C10 (IgG2a), anti-16.5 kDa mAb A68/04G01/C06 (IgG2a), anti-19 kDa mAb A68/25H10/A05 (IgG2a), anti-25–27 kDa mAb A59/05F01/C09 (IgG2a), anti-31–34 kDa mAb A59/10F09/G10 (IgG2a), anti-36–38 kDa mAb A63/05A07/A08 (IgA), anti-89 kDa mAb A53/10B02/A01 (IgG1), anti-R-LPS mAb A68/03F03/D05 (IgG2b), anti-S-LPS mAb A76/12G12/F12 (IgG1) and anti-PG mAb A76/03D06/A09 (IgG3). The mAb specific for PG (A76/03D06/A09) was obtained by fusion of spleen cells of mice infected with *B. melitensis* B115 with the NSO non-secreting myeloma cell line as described by (Cloeckaert *et al.*, 1990), and was called 3D6.

Antisera. Rabbit anti-mouse immunoglobulin serum (RAM) was produced by repeated intradermal injections of 100 µg mouse IgG. Rabbits were injected and bled every two weeks over several months. The best bleedings were pooled.

Peroxidase conjugation. Protein A (Sigma) was conjugated with horseradish peroxidase (Sigma) according to a modification of the method of Nakane & Kawaoi (1974) as described by Dubray & Limet (1987). The protein/enzyme ratio was 1:2.

Latex agglutination immunoassay. This was performed as described previously (Limet *et al.*, 1988). Latex particles were coated with the purified 25–27 kDa major *Brucella* OMP. A 10% (w/v) latex suspension (50 µl) (K109 Rhône-Poulenc) was added to 100 µg of the 25–27 kDa OMP in 0.5 ml of fivefold-diluted glycine-buffered saline (GBS: 0.17 M-NaCl, 0.1 M-glycine and 6 mM-Na₂N₃, pH 9.2) in water. After incubation for 45 min at room temperature, the 25–27 kDa-coated latex suspension was washed three times with 1 ml GBS containing 1% (w/v) BSA (GBS-BSA) and then resuspended in 1 ml GBS-BSA. The latex suspension was sonicated after each washing. For the latex agglutination immunoassay, the 25–27 kDa-coated latex suspension was diluted 20-fold in GBS-BSA containing 0.4% RAM. To determine the optimal dilution of mAb 3D6 to be used in the agglutination inhibition assay, 30 µl of the latex suspension was incubated with 30 µl GBS-BSA and 30 µl of several dilutions in GBS-BSA of mAb 3D6. After 15 min vortex mixing at 37 °C, samples were diluted with 750 µl GBS containing 0.05% Tween 20. The degree of agglutination was determined by counting the non-agglutinated particles in a Multipact Reader (Acade Instruments, Louvain La Neuve, Belgium). The agglutination inhibition assay was performed by preincubating 30 µl of the peptidoglycan solutions at several concentrations in GBS-BSA with 30 µl mAb 3D6 at the appropriate dilution for 30 min at 37 °C with vortex mixing followed by a further 15 min incubation with 30 µl latex suspension in the same conditions. Results were expressed as percentage inhibition of agglutination. In the absence of inhibitor, 50% of latex particles were agglutinated by mAb 3D6.

Immunoblotting techniques. The protein antigens were separated by SDS-PAGE (Laemmli, 1970), in a 2001 vertical electrophoresis unit (LKB-Produkter). After electrophoresis, the proteins were transferred to nitrocellulose by using a Transblot apparatus (BioLyon, Dardilly,

France) at 12 V for 2 h. Unoccupied sites on the nitrocellulose membranes were blocked by 30 min incubation at room temperature with agitation in Tris-buffered saline (TBS: 0.15 M-NaCl, 10 mM-Tris/HCl, pH 7.5) containing 3% BSA. The membranes were then successively incubated overnight at room temperature with hybridoma supernatants diluted 1 in 2 in TBS containing 1% BSA and 0.05% (v/v) Tween 20, for 1 h with RAM and for 1 h with peroxidase-conjugated protein A, diluted 1 in 250 and 1 in 1000, respectively, in the same buffer. Washings between incubation periods were performed with TBS containing 0.05% Tween 20 (Tw-TBS). After two washings in Tw-TBS and two washings in TBS, the blots were developed by incubation at room temperature in a solution of TBS containing 0.06% 4-chloro-1-naphthol (Bio-Rad) and 5 mM-H₂O₂. The reaction was stopped by washing in distilled water.

Thin-section preparation. The method was as described by Lam *et al.* (1987). Samples were fixed in 1 ml 2.5% (v/v) glutaraldehyde in phosphate-buffered saline (PBS) for one night at 4 °C or in 1 ml 4% (v/v) paraformaldehyde buffered with 0.1 M-cacodylate at pH 7.25 for 1 h at room temperature. After fixation, samples were enrobed in 5% (w/v) agarose and washed in buffer. The cores were then dehydrated through a stepwise alcohol series and infiltrated with propylene oxide. The samples were embedded in Epon 812 resin and cured for 24 h at 37 °C. Ultrathin sections were cut with an ultramicrotome (Reichert Jung Ultracut E) and deposited on 200-mesh gold grids.

Immunogold labelling. (i) *Whole-mount incubations.* A 5 µl sample of cell suspension in water (10¹⁰ bacteria ml⁻¹) was deposited on carbon-Formvar-coated 200-mesh copper grids. After being air dried (about 30 min), the grids were incubated in the following reagents: PBS containing 3% (w/v) BSA (PBS-3% BSA) (30 min at 37 °C); ascitic fluid containing the mAb diluted 1 in 50 in PBS containing 0.05% (v/v) Tween 20 (PBS-Tw) (2 h at 37 °C); sheep anti-mouse biotinylated Ig (Amersham) diluted 1 in 200 in PBS-Tw (1 h at room temperature); gold-labelled streptavidin (15 nm) (Amersham) diluted 1 in 20 in PBS-Tw (1 h at room temperature). Washings between incubation periods were performed with 0.15 M-NaCl containing 0.01% Tween 20 (NaCl-Tw). After four washings in NaCl-Tw and four washings in distilled water, the grids were observed in a transmission electron microscope (Philips CM10).

(ii) *Section incubations.* The same procedure was followed as for whole mounts, except that before incubation in PBS-3% BSA the grids were pretreated in 10% (v/v) H₂O₂ in distilled water for 30 min and then washed in distilled water. After immunogold labelling, the sections were stained with 4% (w/v) uranyl acetate in water and 0.4% lead citrate in 0.1 M-NaOH.

mAb isotypes. These were determined by direct latex agglutination immunoassay of the culture supernatants. Latex particles were coated with rat monoclonal anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and polyclonal anti-mouse IgA according to a protocol similar to that described by Limet *et al.* (1988).

Results

Effect of lysozyme treatment on the reactivity of anti-OMP mAbs and mAb 3D6 in Western blots of Brucella OMPs

The effect of lysozyme treatment was examined by immunoblot analysis with the anti-OMP and 3D6 mAbs after SDS-PAGE of sonicated cell extracts of *B. melitensis* B115. More bands of the major OMPs (25–27,

Fig. 1. Immunoblot of untreated (lanes 1, 3, 5, 7) and lysozyme-treated (lanes 2, 4, 6, 8) sonicated cell extracts of *B. melitensis* B115 with anti-25–27 kDa mAb A59/05F01/C09 (lanes 1 and 2), anti-31–34 kDa mAb A59/10F09/G10 (lanes 3 and 4), anti-36–38 kDa mAb A63/05A07/A08 (lanes 5 and 6) and mAb 3D6 (lanes 7 and 8).

31–34 and 36–38 kDa) were revealed in lysozyme-treated sonicated cell extracts than in untreated extracts (Fig. 1). Moreover, bands of higher molecular mass, ranging from 50 to 54 kDa for the anti-25–27 kDa mAb, 54 to 66 kDa for the anti-31–34 kDa mAb and 78 kDa for the anti-36–38 kDa mAb, were only evident after lysozyme treatment. They were also reactive with the mAb 3D6. No bands reacted with mAb 3D6 in sonicated extracts not treated with lysozyme. Lysozyme treatment did not affect the electrophoretic mobility of the minor OMPs (10, 16.5, 19 and 89 kDa) (Fig. 2).

Reactivity of anti-OMP mAbs and mAb 3D6 with E. coli and Y. enterocolitica sonicated cell extracts in Western blots

Species specificity of these mAbs was determined by immunoblot analysis of lysozyme-treated sonicated cell extracts of the S-LPS cross-reacting strains *E. coli* O:157

Fig. 2. Immunoblot of untreated (lanes 1, 3, 5, 7) and lysozyme-treated (lanes 2, 4, 6, 8) sonicated cell extracts of *B. melitensis* B115 with anti-10 kDa mAb A68/07G11/C10 (lanes 1 and 2), anti-16.5 kDa mAb A68/04G01/C06 (lanes 3 and 4), anti-19 kDa mAb A68/25H10/A05 (lanes 5 and 6) and anti-89 kDa mAb A53/10B02/A01 (lanes 7 and 8).

and *Y. enterocolitica* O:9. No bands reacted with mAbs specific for the OMPs, whereas mAb 3D6 revealed bands ranging from 12 to 43 kDa in these extracts (data not shown).

Reactivity of mAb 3D6 with *E. coli* and *Brucella* PG in latex agglutination inhibition immunoassay

Reactivity of mAb 3D6 with *E. coli* and *Brucella* PG was determined by inhibition of agglutination of latex particles coated with the purified 25–27 kDa OMP (latex-25–27 kDa) showing the multiple band pattern in SDS-PAGE (not shown). Both *E. coli* and *Brucella* PG completely inhibited the agglutination of latex-25–27 kDa by mAb 3D6. *Brucella* PG was at least 10 times more inhibitory than *E. coli* PG (Fig. 3).

Fig. 3. Latex agglutination inhibition immunoassay. Agglutination of latex particles coated with 25–27 kDa OMP by mAb 3D6 was inhibited by purified PG of *B. melitensis* B115 (●) and *E. coli* (○).

Localization of epitopes recognized by mAb 3D6 by immunoelectron microscopy

As expected for an anti-PG mAb, no immunogold labelling was observed on the surface of whole cells of *B. melitensis* B115 when mAb 3D6 was used as primary antibody (Fig. 4a). Under the same conditions immunogold labelling was observed with the mAb specific for the 31–34 kDa OMP which recognizes cell-surface exposed epitopes (Fig. 4c). However, when mAb 3D6 was used in thin sections of *B. melitensis* B115 cells, immunogold labelling was observed close to the outer membrane, in the periplasmic space and in the cytoplasm (Fig. 5).

Reactivity of the anti-PG mAb 3D6 and R-LPS- and S-LPS-specific mAbs with *Brucella* OMPs in Western blots

In lysozyme-treated sonicated cell extracts of *B. melitensis* B115, the anti-R-LPS mAb A68/03F03/D05 reacted only with one major band of molecular mass less than 15 kDa (Fig. 6). With the S-LPS-specific mAb A76/12G12/F12, the classical profile was observed: a smear ranging in molecular mass from 30 to 50 kDa. Multiple major bands were evident when using mAbs specific for the major OMPs ranging from 25 to 27 kDa (three major bands) for the anti-25–27 kDa mAb, 26 to 34 kDa (five to six bands) for the anti-31–34 kDa mAb and 36 to 38 kDa (three major bands) for the anti-36–38 kDa mAb. Bands of higher molecular mass also reacted with these mAbs, at 50 kDa for the anti-25–27 kDa mAb, 54 kDa for the anti-31–34 kDa mAb and 78 kDa for the anti-36–38 kDa mAb. All these bands except for the lowest band of at least the 25–27 and 36–38 kDa OMPs were also revealed by the anti-PG mAb. These bands were not detected by the R-LPS- and S-LPS-specific mAbs.

Fig. 4. Immunogold labelling of whole cells of *B. melitensis* B115 by the anti-PG mAb 3D6 (a), negative control (unrelated to *Brucella*) mAb (b), and anti-31–34 kDa mAb A59/10F09/G10 (c). Bars, 0.5 μ m.

Fig. 5. Immunogold labelling of thin sections of cells of *B. melitensis* B115 with negative control mAb (a) and anti-PG mAb 3D6 (b). Bars, 0.25 µm.

Discussion

mAb 3D6 recognized a large number of bands in Western blots of lysozyme-treated sonicated cell extracts of *B. melitensis*. The reactivity of mAb 3D6 in Western blots was largely dependent on lysozyme treatment. In contrast to the *Brucella* OMP-specific mAbs, mAb 3D6 reacted with bands in lysozyme-treated sonicated cell extracts of *E. coli* O:157 and *Y. enterocolitica* O:9 (data not shown). Immunogold labelling with mAb 3D6 was only observed on ultrathin sections in close association with the outer membrane and in the periplasmic space, in accordance with the known localization of polymerized PG. All these results, and the fact that this mAb bound specifically to *E. coli* and *Brucella* PG in a latex agglutination inhibition immunoassay, indicate that this mAb is specific for PG subunits. The immunogold labelling in the cytoplasm could be explained by the

Fig. 6. Immunoblot of lysozyme-treated sonicated cell extracts of *B. melitensis* B115 with anti-R-LPS mAb A68/03F03/D05 (lane 1), anti-S-LPS mAb A76/12G12/F12 (lane 2), anti-25–27 kDa mAb A59/05F01/C09 (lane 3), anti-31–34 kDa mAb A59/10F09/G10 (lane 4), anti-36–38 kDa mAb A63/05A07/A08 (lane 5), and anti-PG mAb 3D6 (lane 6).

cytoplasmic steps of biosynthesis of PG precursors (Pellon, 1990). The PG of *Brucella* has the same composition as that of *E. coli* (Lopez-Merino *et al.*, 1976; Mardarowicz, 1966). However, mAb 3D6 reacted preferentially with *Brucella* PG. This could be due to structural differences between *E. coli* and *Brucella* PG, possibly due to *O*-acetylation (Blundell *et al.*, 1980; Dougherty, 1983; Gmeiner & Kroll, 1981; Lear & Perkins, 1983; Rosenthal *et al.*, 1982, 1983; Swim *et al.*, 1983) or the degree of peptide cross-linking (Amano & Williams, 1983; Lear & Perkins, 1983), which confer a greater resistance of PG to lysozyme hydrolysis.

The large differences between immunoblots of untreated and lysozyme-treated sonicated cell extracts revealed by mAbs specific for the major OMPs suggest that large amounts of these proteins are tightly associated, presumably covalently, to the PG. Close association of the PG layer with the outer membrane has already been demonstrated by electron microscopy (Dubray, 1973). The higher-molecular-mass forms of the three major OMPs only occurred after lysozyme treatment. PG-associated OMPs would be released by lysozyme treatment, with one, two, etc., murein subunits remaining attached, giving them an increasingly lower electrophoretic mobility. Similar phenomena have also been described for other Gram-negative bacteria, including Braun's lipoprotein of *E. coli* (Wensink & Witholt, 1981) and the major OMPs of *Rhizobium leguminosarum* (de Maagd *et al.*, 1989a, b) and *Legionella pneumophila* (Butler & Hoffman, 1990) and for the M protein of group A streptococci (Fischetti *et al.*, 1985; Pancholi & Fischetti, 1988). The high-molecular-mass bands in immunoblots of lysozyme-treated sonicated cell extracts, ranging from 50 to 54 kDa reacting with the anti-25–27 kDa mAb, 54 to 66 kDa with the anti-31–34 kDa mAb and 78 kDa with the anti-36–38 kDa mAb, probably represent dimeric forms of each major OMP where the monomers are linked together by PG. Lysozyme treatment seems not to be necessary to release the major OMPs from the cell wall. The percentage of the major OMPs associated with PG has yet to be determined.

The results observed with the anti-36–38 kDa mAb are clear. In sonicated cell extracts not treated with lysozyme only one major band at 36 kDa is observed. In lysozyme-treated sonicated cell extracts three bands react with the anti-36–38 kDa mAb: the 36 kDa band and bands at 37.5 and 38 kDa. The latter two also react with the anti-PG mAb but not with mAbs specific for R-LPS and S-LPS. Thus the multiple banding pattern observed for the major 36–38 kDa OMP is essentially due to PG subunit residues associated with this protein. In contrast to the 36–38 kDa OMP, multiple bands were reactive in non-lysozyme-treated sonicated cell extracts with the

anti-25–27 kDa and anti-31–34 kDa mAbs (Fig. 1). Association of PG subunits with the major 25–27 kDa protein is not the only cause of heterogeneity observed in Western blots. It was suggested by Dubray & Charriaut (1983) that the 25–27 kDa OMP could possibly be a glycoprotein containing 1,2-diol groups since this protein was detected by silver staining after periodic acid oxidation. We obtained IgG3 mAbs specific for the 25–27 kDa OMP which recognized periodate-sensitive epitopes that were not sensitive to pepsin treatment and probably recognize the oligosaccharide portion of this OMP (unpublished observations). Thus the multiple band pattern observed for the 25–27 kDa OMP could also be due to variation in the size of the oligosaccharide portion. For the 31–34 kDa OMP the results are more difficult to interpret. The S-LPS- and R-LPS-specific mAbs did not detect a multiple banding pattern in the lysozyme-treated sonicated *Brucella* cell extracts. Thus, differences in electrophoretic mobility of the major *Brucella* OMP bands in SDS-PAGE are not due to association of S-LPS or R-LPS molecules.

There is no evidence of association of the minor OMPs of molecular mass 10, 16.5, 19, and 89 kDa with PG. We also did not detect the low-molecular-mass lipoprotein linked to PG described by others (Gomez-Miguel & Moriyon, 1986). The anti-PG mAb reacted with no other bands than those which bound the anti-major OMP mAbs. These results are in contradiction to those of Gomez-Miguel & Moriyon (1986), who suggested that the lipoprotein is the only major protein covalently linked to the PG.

In conclusion, our results clearly demonstrate the close association between a proportion of major OMPs and PG. This association explains the heterogeneity observed in Western blots for the 36–38 kDa OMP after lysozyme treatment and part of the multiple banding pattern observed for the 25–27 and 31–34 kDa OMPs.

We are most grateful to J. M. Verger and M. Grayon for culture and control of bacterial strains. We thank G. Bézard, A. Van Rompaye, J. Van Broeck and J. C. Nicolle for expert technical assistance.

A. Cloeckaert was supported by the Institut National pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture.

References

- AMANO, K.-I. & WILLIAMS, J. C. (1983). Peptidoglycan of *Legionella pneumophila*: apparent resistance to lysozyme hydrolysis correlates with a high degree of peptide cross-linking. *Journal of Bacteriology* **153**, 520–526.
- BLUNDELL, J. K., SMITH, G. J. & PERKINS, H. R. (1980). The peptidoglycan of *Neisseria gonorrhoeae*: *O*-acetyl groups and lysozyme sensitivity. *FEMS Microbiology Letters* **9**, 259–261.
- BUTLER, C. A. & HOFFMAN, P. S. (1990). Characterization of a major 31-kilodalton peptidoglycan-bound protein of *Legionella pneumophila*. *Journal of Bacteriology* **172**, 2401–2407.

- CLOECKAERT, A., DE WERGIFOSSE, P., DUBRAY, G. & LIMET, J. N. (1990). Identification of seven surface-exposed *Brucella* outer membrane proteins by use of monoclonal antibodies: immunogold labeling for electron microscopy and enzyme-linked immunosorbent assay. *Infection and Immunity* **58**, 3980–3987.
- DOUGHERTY, J. T. (1983). Synthesis and modification of the peptidoglycan in *Neisseria gonorrhoeae*. *FEMS Microbiology Letters* **17**, 51–53.
- DOUGLAS, J. T., ROSENBERG, E. Y., NIKAIKO, H., VERSTRAETE, D. R. & WINTER, A. J. (1984). Porins of *Brucella* species. *Infection and Immunity* **44**, 16–21.
- DUBRAY, G. (1973). Le peptidoglycane des *Brucella*: mise en évidence d'une structure à triple feuillet. *Comptes Rendus de l'Académie des Sciences Paris* **277**, 2281–2283.
- DUBRAY, G. (1981). *Etude ultrastructurale et biochimique des enveloppes des bactéries du genre Brucella*. PhD thesis, Université de Paris-Sud.
- DUBRAY, G. (1987). Protective antigens in brucellosis. *Annales de l'Institut Pasteur/Microbiologie* **138**, 84–86.
- DUBRAY, G. & BÉZARD, G. (1980). Isolation of three protective cell-wall antigens of *Brucella abortus* in experimental brucellosis in mice. *Annales de Recherches Vétérinaires* **11**, 367–373.
- DUBRAY, G. & CHARRIAUT, C. (1983). Evidence of three major polypeptide species and two major polysaccharide species in the *Brucella* outer membrane. *Annales de Recherches Vétérinaires* **14**, 311–318.
- DUBRAY, G. & LIMET, J. (1987). Evidence of heterogeneity of lipopolysaccharides among *Brucella* biovars in relation to A and M specificities. *Annales de l'Institut Pasteur/Microbiologie* **138**, 27–37.
- DUBRAY, G. & PLOMMET, M. (1976). Structure et constituants des *Brucella*. Caractérisation des fractions et propriétés biologiques. *Developments in Biological Standardization* **31**, 68–91.
- FISCHETTI, V. A., JONES, K. F. & SCOTT, J. R. (1985). Size variation of the M protein in group A streptococci. *Journal of Experimental Medicine* **161**, 1384–1401.
- GMEINER, J. & KROLL, H.-P. (1981). Murein biosynthesis and O-acetylation of N-acetylmuramic acid during the cell-division cycle of *Proteus mirabilis*. *European Journal of Biochemistry* **117**, 171–177.
- GOMEZ-MIGUEL, M. J. & MORIYON, I. (1986). Demonstration of a peptidoglycan-linked lipoprotein and characterization of its trypsin fragment in the outer membrane of *Brucella* spp. *Infection and Immunity* **53**, 678–684.
- GOMEZ-MIGUEL, M. J., MORIYON, I. & LOPEZ, J. (1987). *Brucella* outer membrane lipoprotein shares antigenic determinants with *Escherichia coli* Braun lipoprotein and is exposed on the cell surface. *Infection and Immunity* **55**, 258–262.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LAM, J. S., LAM, M. Y. C., MACDONALD, L. A. & HANCOCK, R. E. W. (1987). Visualization of *Pseudomonas aeruginosa* O antigens by using a protein A-dextran-colloidal gold conjugate with both immunoglobulin G and immunoglobulin M monoclonal antibodies. *Journal of Bacteriology* **169**, 3531–3538.
- LEAR, A. L. & PERKINS, H. R. (1983). Degrees of O-acetylation and cross-linking of the peptidoglycan of *Neisseria gonorrhoeae* during growth. *Journal of General Microbiology* **129**, 885–888.
- LIMET, J. N., BERBINSCHI, A., CLOECKAERT, A., CAMBIASO, C. L. & MASSON, P. L. (1988). Longitudinal study of brucellosis in mice by immunoassay of lipopolysaccharide-related antigens in blood and urine. *Journal of Medical Microbiology* **26**, 37–45.
- LOPEZ-MERINO, A., ASSELINEAU, J., SERRE, A., ROUX, J., BASCOUL, S. & LACAVE, C. (1976). Immunization by an insoluble fraction extracted from *Brucella melitensis*: immunological and chemical characterization of the active substances. *Infection and Immunity* **13**, 311–321.
- DE MAAGD, R. A., DE RIJK, R., MULDER, I. H. M. & LUGTENBERG, B. J. J. (1989a). Immunological characterization of *Rhizobium leguminosarum* outer membrane antigens by use of polyclonal and monoclonal antibodies. *Journal of Bacteriology* **171**, 1136–1142.
- DE MAAGD, R. A., WIENTJES, F. B. & LUGTENBERG, B. J. (1989b). Evidence for divalent cation (Ca²⁺)-stabilized oligomeric proteins and covalently bound protein-peptidoglycan complexes in the outer membrane of *Rhizobium leguminosarum*. *Journal of Bacteriology* **171**, 3989–3995.
- MARDAROWICZ, C. (1966). Isolierung und Charakterisierung des Murein-Sacculus von *Brucella*. *Zeitschrift für Naturforschung* **21b**, 1006–1007.
- MORIYON, I. & BERMAN, D. T. (1983). Isolation, purification, and partial characterization of *Brucella abortus* matrix protein. *Infection and Immunity* **39**, 394–402.
- NAKANE, P. K. & KAWAOI, A. (1974). Peroxidase-labelled antibody, a new method of conjugation. *Journal of Histochemistry and Cytochemistry* **22**, 1084–1091.
- PANCHOLI, V. & FISCHETTI, V. A. (1988). Isolation and characterization of the cell-associated region of group A streptococcal M6 protein. *Journal of Bacteriology* **170**, 2618–2624.
- PELLON, G. (1990). Biosynthesis of peptidoglycans from eubacterial cell envelopes. *Bulletin de l'Institut Pasteur* **88**, 203–236.
- ROSENTHAL, R. S., BLUNDELL, J. K. & PERKINS, H. R. (1982). Strain-related differences in lysozyme sensitivity and extent of O-acetylation of gonococcal peptidoglycan. *Infection and Immunity* **37**, 826–829.
- ROSENTHAL, R. S., FOLKENING, W. J., MILLER, D. R. & SWIM, S. C. (1983). Resistance of O-acetylated gonococcal peptidoglycan to human peptidoglycan-degrading enzymes. *Infection and Immunity* **40**, 903–911.
- SANTOS, J. M., VERSTRAETE, D. R., PERERA, V. Y. & WINTER, A. J. (1984). Outer membrane proteins from rough strains of four *Brucella* species. *Infection and Immunity* **46**, 188–194.
- SWIM, S. C., GFELL, M. A., WILDE, C. E., III & ROSENTHAL, R. S. (1983). Strain distribution in extents of lysozyme resistance and O-acetylation of gonococcal peptidoglycan determined by high-performance liquid chromatography. *Infection and Immunity* **42**, 446–452.
- VERSTRAETE, D. R. & WINTER, A. J. (1984). Comparison of sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles and antigenic relatedness among outer membrane proteins of 49 *Brucella abortus* strains. *Infection and Immunity* **46**, 182–187.
- VERSTRAETE, D. R., CREASY, M. T., CAVENEY, N. T., BALDWIN, C. L., BLAB, M. W. & WINTER, A. J. (1982). Outer membrane proteins of *Brucella abortus*: isolation and characterization. *Infection and Immunity* **35**, 979–989.
- WENSINK, J. & WITHOLT, B. (1981). Identification of different forms of the murein-bound lipoprotein found in isolated outer membranes of *Escherichia coli*. *European Journal of Biochemistry* **113**, 349–357.