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# *Brucella* Outer Membrane Lipoprotein Shares Antigenic Determinants with *Escherichia coli* Braun Lipoprotein and Is Exposed on the Cell Surface

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**In an enzyme-linked immunosorbent assay (ELISA), purified *Brucella abortus* and *Escherichia coli* peptidoglycan-linked lipoproteins gave a strong cross-reaction with sera from rabbits hyperimmunized with the heterologous lipoprotein. When smooth *E. coli* cells were used as ELISA antigens, the immunological cross-reaction was not observed unless the cells were treated to remove lipopolysaccharide and other outer membrane components. In contrast, intact cells from smooth strains of *B. abortus* and *Brucella melitensis* bound anti-lipoprotein immunoglobulin G, and the controls performed by ELISA showed that this reaction was not due to antibodies to the lipopolysaccharide, group 3 outer membrane proteins, or porins. Electron microscopy of cells labeled with antilipoprotein serum and protein A-colloidal gold showed specific labeling of smooth cells from both *B. abortus* and *B. melitensis*, even though unspecific labeling by nonimmune serum was observed with rough *B. abortus*. These results confirm the close similarity between *E. coli* and *Brucella* peptidoglycan-linked lipoproteins and show that, in contrast to *E. coli*, the lipoprotein of *B. abortus* and *B. melitensis* is partially exposed on the surface of smooth cells.**

The outer membrane of smooth and rough *Brucella* species contains three groups of major proteins which can be obtained by the Sarkosyl-Zwittergent extraction method (22, 29, 32). In contrast to group 1 which remains largely uncharacterized, group 2 proteins have been identified as matrix proteins (23) with porin activity (8), and group 3 proteins probably represent the *Brucella* counterparts of *Escherichia coli* OmpA (32). In addition, we have shown recently that the cell envelopes of the rough mutant *Brucella abortus* 45/20 and *Brucella ovis* REO198 contain a low-molecular-weight lipoprotein bound to the peptidoglycan by a trypsin-sensitive linkage (14). In the same work, it was shown that the trypsin fragment of the *B. abortus* lipoprotein is similar to *E. coli* Braun lipoprotein in mobility in sodium dodecyl sulfate (SDS)-polyacrylamide gels, molecular weight, presence of linked fatty acids, isoelectric point, and overall amino acid composition (14). These data suggest that both lipoproteins also share some additional features such as parts of the tertiary structure or the extent to which they are inserted in the outer membrane. Results of experiments concerning these questions are presented in this report.

## MATERIALS AND METHODS

**Bacterial strains.** The following strains were used in this work: *B. abortus* 19 (smooth, attenuated), *B. abortus* 45/20 (rough, avirulent), *Brucella melitensis* 16M (smooth, virulent), *B. melitensis* 115 (rough, avirulent), and *B. ovis* REO198 (avirulent). Other characteristics of these strains have been presented elsewhere (1, 6, 16, 29, 32). *E. coli* B MH1283 (rough) and *E. coli* CUN237 (smooth) have been used in previous work (14, 25). To ensure the smoothness of *B. melitensis* 16M, the strain was inoculated into guinea pigs, recovered from the spleen 7 days later, and kept in skim milk (Difco Laboratories, Detroit, Mich.) at −75°C.

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**Cell fractionation and lipoprotein extraction.** Lipoproteins were obtained following the modification of the protocol of Braun and Sieglin (3) described previously (14). Briefly, exponential-phase cells of *B. abortus* 45/20, *B. ovis* REO198, or *E. coli* B were disrupted by ballistic disintegration, and the cell envelope fraction was obtained by ultracentrifugation. Cell envelopes were suspended in distilled $H_2O$ and extracted with continuous magnetic stirring for 20 min at 100°C in 4% SDS–10 mM Tris hydrochloride (pH 7.5). After cooling to room temperature, the SDS-insoluble residue was recovered by centrifugation (40,000 × *g*, 30 min, 15°C), resuspended again in distilled $H_2O$, and reextracted two more times under the same conditions. The final SDS-insoluble pellet was washed extensively with acetone-water (6:1, vol/vol), suspended in 10 mM Tris hydrochloride (pH 8.3), and digested with trypsin (1 mg/10 mg of protein) for 3 h at 37°C. Proteolysis was stopped by cooling the mixture to 4°C. The trypsin-resistant fraction was removed (40,000 × *g*, 30 min at 4°C), and the trypsin fragment of the lipoprotein was precipitated by the addition of 5 volumes of acetone to the supernatant (−20°C overnight). Finally, the precipitate was collected by centrifugation, suspended in a minimal amount of 2% SDS in 10 mM Tris hydrochloride (pH 8), and purified by gel filtration on Bio-Gel P-60 (Bio-Rad Laboratories, Richmond, Calif.).

**LPS and outer membrane protein preparations.** *B. melitensis* 115 rough lipopolysaccharide (LPS) was extracted from acetone-dried cells by the petroleum ether-chloroform-phenol method (12); since these extracts contained less than 1% protein, they were used without further purification. To solubilize rough LPS for enzyme-linked immunosorbent assays (ELISAs), the antigen was coupled to bovine serum albumin as described by Galanos et al. (13).

*B. ovis* REO198 matrix or porin proteins were obtained from cell envelopes extracted with SDS at 60°C by extraction with either NaCl or $MgCl_2$ in Tris-SDS as described previously (23, 25). Group 3 outer membrane proteins were

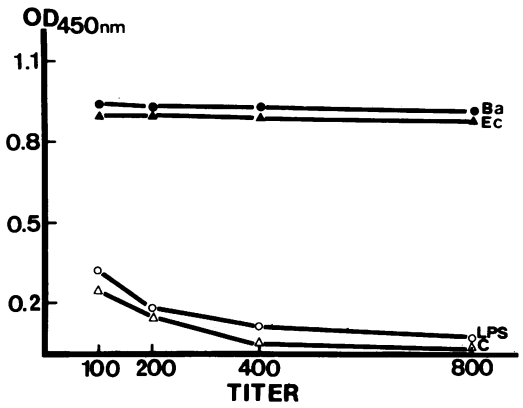


FIG. 1. Analysis by ELISA of the immunological cross-reactivity between *B. abortus* (Ba) and *E. coli* (Ec) lipoproteins with the anti-*B. abortus* lipoprotein serum. The purified trypsin fragment of both lipoproteins was used as the antigen. Controls were normal rabbit serum with *B. abortus* lipoprotein (C) and antilipoprotein serum with *B. melitensis* 115 rough LPS (LPS). $OD_{450nm}$, Optical density at 450 nm.

obtained from *B. ovis* REO198. Briefly, cell envelopes were digested with lysozyme, the outer membrane proteins were extracted with detergents, and group 3 proteins were partially purified by gel filtration on Sephacryl S-300 in the presence of SDS.

**Analytical and immunological methods.** Protein was determined colorimetrically following the modification of the Lowry method described by Dulley and Grieve (10), using bovine serum albumin as the standard.

Antisera to purified *B. abortus* 45/20 lipoprotein and *E. coli* B lipoprotein were obtained from rabbits hyperimmunized with the corresponding purified preparations (14). A serum against whole cell envelopes of *B. melitensis* 115 was raised in rabbits following the hyperimmunization schedule described by Smyth et al. (31).

ELISAs were carried out with either purified lipoprotein or whole cells as antigens. The lipoproteins were attached to polystyrene plates with 60 mM carbonate (pH 9.6) as a coating buffer (5 μg of protein per ml) and overnight incubation at 37°C. When whole cells were used, they were grown on 2% glycerol-potato agar slants for 18 to 36 h at 37°C and washed off the slant with saline. With the virulent strain *B. melitensis* 16M, 0.5% phenol in saline was used followed by inactivation at 37°C. For coupling to polystyrene plates, bacteria were suspended in carbonate buffer at a final optical density at 600 nm of 0.130 and incubated at 37°C overnight. After removal of uncoupled cells with several washes of 0.05% Tween 20 in 20 mM phosphate-buffered saline (pH 7.2), the assay was performed as described elsewhere (17) with peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (heavy- and light-chain specificity) of commercial origin (Nordic Laboratories, Tilburg, Holland) and $H_2O_2$–5-amino-2-hydroxybenzoic acid as the developing substrate. Western blots were performed as described elsewhere (14).

**Immunogold electron microscopy.** The method described by Vos-Scheperkeuter et al. (33) was used with minor modifications. Viable cells, grown as described for the ELISA, were washed twice with sterile saline at 4°C and fixed by overnight incubation in 1% $OsO_4$ in M63 minimal salt solution (19) at 4°C. The $OsO_4$ was removed with several washes of 20 mM phosphate-buffered saline (pH 7.2), and cells were resuspended in the same buffer solution at a final optical density of 0.650 at 600 nm. A small volume (150 to 250 μl) of this suspension was mixed with an equal volume of antilipoprotein serum in a 2-ml microcentrifuge plastic cup, and the mixture was incubated for 1 h at 37°C. The cells were then pelleted by centrifugation (3 min at 12,000 × *g*; Fisher microcentrifuge 235A), washed repeatedly with 20 mM phosphate-buffered saline (pH 7.2), and incubated in the same plastic cups with 25 to 50 μl of protein A-colloidal gold (see below) for 30 min at room temperature. The unbound protein A-colloidal gold was removed with several phosphate-buffered saline washes, and the cells were resuspended in 50 to 75 μl of the same buffer solution. Controls were performed in the same way but with normal rabbit serum instead of antilipoprotein serum.

Colloidal gold was prepared by the method of Frens (11), and protein A was obtained by affinity chromatography on Sepharose 4B-human IgG of culture supernatants of the protein A-releasing mutant *Staphylococcus aureus* A676 (27). To couple protein A to colloidal gold, polyethylene glycol was used by the method of Horisberg et al. (15) as modified by Roth et al. (28).

For electron microscopy, the cells were spread on Formvar–carbon-coated grids which were examined in a Zeiss EM-10 CR apparatus.

## RESULTS

The immunological cross-reactivity of *B. abortus* and *E. coli* lipoprotein was examined first by ELISA with the purified trypsin fragments of both lipoproteins as antigens. The results of this analysis showed that the serum to *B. abortus* lipoprotein gave a strong reaction with both antigens, and since the control serum only produced background optical density, it was also demonstrated that this reaction was specific (Fig. 1). Similar results were obtained with the serum to *E. coli* lipoprotein (data not shown) or with the lipoprotein from *B. ovis* (data not shown). In addition, in the same set of experiments it was observed that the antilipoprotein serum did not react with the rough LPS of *B. melitensis* 115 (Fig. 1) (the rough LPS of *B. abortus* and *B.*

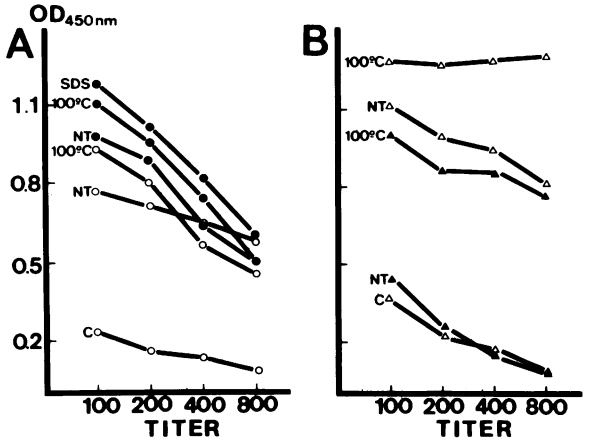


FIG. 2. ELISA with anti-*B. abortus* lipoprotein serum and whole cells of *B. abortus* (A) and *E. coli* (B). The strains used were *B. abortus* 45/20 (○), *B. abortus* 19 (●), *E. coli* B (△) and *E. coli* CUN237 (◀). Intact viable cells were subjected to the following treatments before coupling to the solid phase: NT, no treatment; 100°C, boiling in water; SDS, boiling in 0.2% SDS. Controls (C) were performed with nonimmune serum. $OD_{450nm}$, Optical density at 450 nm.

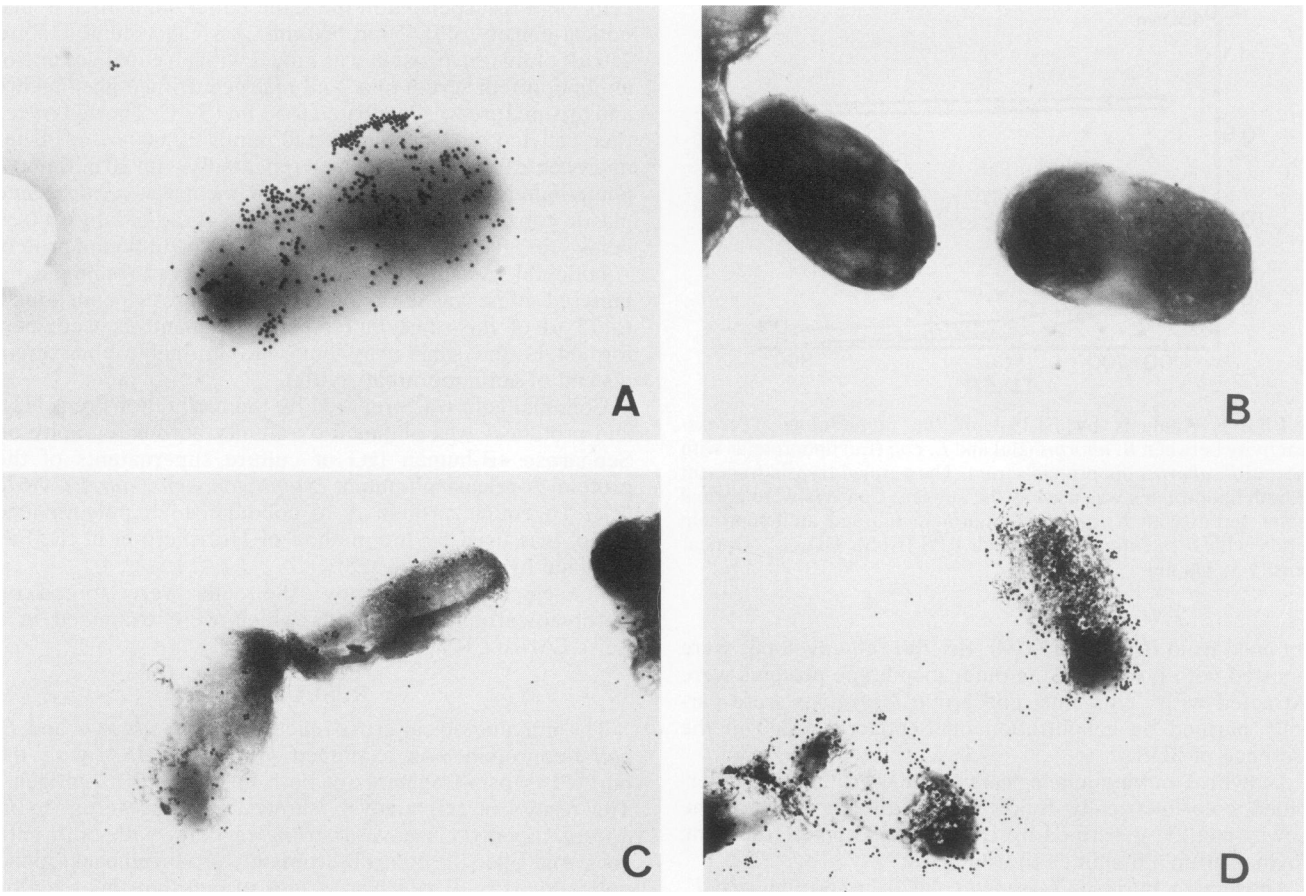


FIG. 3. Immunoelectron microscopy of *Brucella* cells labeled with rabbit sera and protein A-colloidal gold. The results of the following combinations of sera and bacterial strains are presented: (A) *B. abortus* 19 (smooth) with antilipoprotein serum; (B) *B. abortus* 19 with normal serum; (C) *B. abortus* 45/20 (rough) with normal serum; (D) *B. melitensis* 16M (smooth) with antilipoprotein serum.

*melitensis* give immunological identity in gel precipitation tests [5, 21]).

The immunological cross-reactivity was also examined with the same antilipoprotein serum and rough and smooth cells of both *E. coli* and *B. abortus*. The results of this second set of experiments are presented in Fig. 2. In *B. abortus* both the strain 45/20 (rough) and the strain 19 (smooth) cells bound antibody specifically (Fig. 2A). Controls performed by Western blot showed that the serum did not contain antibody to group 3 proteins, although a weak reaction was observed with the porin preparations. However, absorption of the antiporin antibody (three times with 1 mg of protein per ml of serum) with *B. ovis* porin proteins did not change the titers obtained with the whole cells. Finally, and in contrast with the observations made with *B. abortus*, the rough strain *E. coli* B MH1283 but not the smooth strain *E. coli* CUN237 bound antibodies to *B. abortus* lipoprotein (Fig. 2B).

Besides showing a cross-reaction between the native lipoproteins of *E. coli* and *B. abortus*, the above results suggested that in *B. abortus* the lipoprotein was exposed on the cell surface of smooth cells. To test this hypothesis, we studied to what extent removal of LPS and other outer membrane components would increase the amount of antilipoprotein antibody bound by the smooth strains of both *B. abortus* and *E. coli*. To achieve such removal, smooth and rough cells were suspended in distilled water, boiled for 30 min, and recovered by centrifugation, a treatment which removed large amounts of both LPS and outer membrane proteins (7; data not shown). This removal caused a strong increase in the amount of antibody bound by the smooth *E. coli* cells (Fig. 2B). In contrast, only a small increase was observed with either the smooth or rough *B. abortus* (Fig. 2A). A more drastic treatment of the smooth *B. abortus* cells with detergent (boiling in 0.2% SDS for 2 min) produced a further increase in the amount of antibody bound by the *Brucella* strains (Fig. 2A, SDS).

The smooth and rough *B. abortus* strains were also examined by electron microscopy after being labeled with antilipoprotein serum and protein A-colloidal gold. Antilipoprotein IgG but not IgG from normal serum bound to the surface of *B. abortus* 19 (smooth) (Fig. 3A and B). Even though more gold granules were observed on *B. abortus* 45/20 (rough) cells labeled with antilipoprotein IgG, unspecific labeling was also detected with this strain (Fig. 3C). Nonspecific binding of IgG to rough *Brucella* species has been observed by other investigators (30).

Finally, similar experiments were carried out with *B. melitensis*. First, it was shown by ELISA that the serum prepared against *B. melitensis* 115 reacted with purified *B. abortus* lipoprotein and that the antilipoprotein serum reacted with whole cells of both *B. melitensis* 115 and *B. melitensis* 16M (data not shown). As was observed with the smooth strain *B. abortus* 19, electron microscopy of *B. melitensis* 16M labeled with antilipoprotein serum plus protein A-colloidal gold showed specific surface labeling (Fig.

3D). Controls with negative serum were also negative in this case (data not shown).

## DISCUSSION

The data presented in this work show that antisera to *B. abortus* and *E. coli* peptidoglycan-linked lipoproteins reacted with the heterologous purified antigen and that the cross-reaction was also observed with *E. coli* whole cells which had been treated to remove outer membrane components. Although it has been shown that *E. coli* and Brucella LPSs share antigenic determinants in their lipid A (20), the cross-reaction reported here cannot be due to LPS contamination since the antiserum used did not contain antibodies to purified *B. melitensis* 115 LPS which could be shown by ELISA.

Even though a close similarity has been shown for the lipoproteins of several enterobacteria, *Proteus* species is a significant exception (26). Therefore, both the results of the biochemical characterization (14) and the immunological cross-reaction suggest a relationship between *E. coli* and *Brucella* lipoproteins closer than the one which could be expected from their taxonomical position. This similarity, which probably reflects a similar function for both lipoproteins, could account for the results of a previous work in which it was found that *B. abortus* protein-peptidoglycan sacculi bind *E. coli* matrix (porin) proteins in amounts similar to those bound by the homologous sacculi (24). Yamada and Mizushima (34, 35) have demonstrated that the in vivo lipoprotein-matrix protein interactions (4) are reestablished during in vitro homologous reconstitutions, and therefore it seems very likely that interactions between *Brucella* lipoprotein and *E. coli* matrix protein contribute to the heterologous peptidoglycan-matrix protein binding. Obviously, a close similarity between the peptidoglycan-linked lipoproteins would favor such interactions.

Despite the similarity between both lipoproteins, there was a remarkable difference with respect to their exposure on the cell surface. The immunoelectron microscopy experiments performed with whole cells showed that antilipoprotein IgG reacted with antigenic determinants present on the surface of smooth *B. melitensis* and *B. abortus*. In contrast, such a reaction was not observed with intact smooth *E. coli* cells and the anti-*Brucella* lipoprotein serum. Although this last observation could be explained if we assume that the antigenic determinants shared by both lipoproteins are not exposed on *E. coli*, it has been shown by immunological methods that *E. coli* lipoprotein is not exposed on the surface of smooth cells (2, 18). It has to be stressed that the results obtained with *Brucella* species cannot be due to anti-smooth LPS antibodies since the serum was prepared with the lipoprotein of a rough strain. Moreover, the controls showed that the serum used did not contain antibodies to the rough LPS.

The interpretation that *Brucella* lipoprotein but not *E. coli* lipoprotein is partially exposed on the surface is strengthened by the results obtained by ELISA with whole cells, either intact or after treatments removing outer membrane components. While *E. coli* smooth cells reacted only after such treatments, smooth *Brucella* cells reacted both before and after. Thus, it must be concluded that, in contrast to *E. coli*, in *Brucella* smooth cells the LPS *O*-polysaccharide chain does not hinder the reaction of IgG antibodies with the lipoprotein. This conclusion does not necessarily concern the structure of *Brucella* lipoprotein, since it has been shown by electron microscopy that the layer corresponding to the polysaccharide moiety of the LPS is less thick in *Brucella* smooth cells than in *E. coli* smooth cells (9). However, it remains to be determined whether the reaction observed was due to the bound form of the lipoprotein present in *Brucella* outer membrane (14) or to a possible free form.

Finally, the results of this work demonstrate that antibodies specific for the lipoprotein of *B. abortus* 45/20 react with antigenic components of rough and smooth *B. melitensis*. Conversely, antisera to whole cell envelopes of *B. melitensis* 115 reacted with purified *B. abortus* 45/20 lipoprotein. These results strongly suggest that, in addition to *B. abortus* and *B. ovis* (14), a peptidoglycan-linked lipoprotein with characteristics close to those of *E. coli* Braun lipoprotein is also present in *B. melitensis*.

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