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Brucella-Salmonella Lipopolysaccharide Chimeras Are Less Permeable to Hydrophobic Probes and More Sensitive to Cationic Peptides and EDTA than Are Their Native *Brucella* sp. Counterparts

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A rough (R) *Brucella abortus* 45/20 mutant was more sensitive to the bactericidal activity of polymyxin B and lactoferricin B than was its smooth (S) counterpart but considerably more resistant than *Salmonella montevideo*. The outer membrane (OM) and isolated lipopolysaccharide (LPS) of *S. montevideo* showed a higher affinity for these cationic peptides than did the corresponding *B. abortus* OM and LPS. We took advantage of the moderate sensitivity of R *B. abortus* to cationic peptides to construct live R *B. abortus*–S-LPS chimeras to test the activities of polymyxin B, lactoferricin B, and EDTA. Homogeneous and abundant peripheral distribution of the heterologous S-LPS was observed on the surface of the chimeras, and this coating had no effect on the viability or morphology of the cells. When the heterologous LPS corresponded to the less sensitive bacterium S *B. abortus* S19, the chimeras were more resistant to cationic peptides; in contrast, when the S-LPS was from the more sensitive bacterium *S. montevideo*, the chimeras were more susceptible to the action of peptides and EDTA. A direct correlation between the amount of heterologous S-LPS on the surface of chimeric *Brucella* cells and peptide sensitivity was observed. Whereas the damage produced by polymyxin B in *S. montevideo* and *B. abortus–S. montevideo* S-LPS chimeras was manifested mainly as OM blebbing and inner membrane rolling, lactoferricin B caused inner membrane detachment, vacuolization, and the formation of internal electron-dense granules in these cells. Native S and R *B. abortus* strains were permeable to the hydrophobic probe *N*-phenyl-1-naphthylamine (NPN). In contrast, only reduced amounts of NPN partitioned into the OMs of the *S. montevideo* and *B. abortus–S. montevideo* S-LPS chimeras. Following peptide exposure, accelerated NPN uptake similar to that observed for *S. montevideo* was detected for the *B. abortus–S. montevideo* LPS chimeras. The partition of NPN into native or EDTA-, polymyxin B-, or lactoferricin B-treated LPS micelles of *S. montevideo* or *B. abortus* mimicked the effects observed with intact cells, and this was confirmed by using micelle hybrids of *B. abortus* and *S. montevideo* LPSs. The results showed that LPS is the main cause of *B. abortus*' resistance to bactericidal cationic peptides, the OM-disturbing action of divalent cationic chelants, and OM permeability to hydrophobic substances. It is proposed that these three features are related to the ability of *Brucella* bacteria to multiply within phagocytes.

The genus *Brucella* comprises gram-negative, facultative intracellular pathogens that produce disease in a large number of mammals, including humans (8). The outer membrane (OM) of *Brucella* organisms shares many structural features with the OM of their close relatives in the genera *Ochrobactrum*, *Bartonella*, *Agrobacterium*, *Rhizobium*, and *Phyllobacterium*, all of which are eukaryotic-cell-associated α-2 proteobacteria (27, 31). However, the *Brucella* OM differs from that of other pathogenic groups (6, 25–27), including several facultative intracellular bacteria, such as *Salmonella* or *Shigella* spp. For instance, both phosphatidylcholine and ornithine lipids constitute a major part of the *Brucella* OM lipids (6, 42). The lipopolysaccharide (LPS) molecule contains fatty acids of up to 30 carbon atoms which may span the OM and comparatively small quantities of negatively charged 3-deoxy-D-manno-octulosonic acid and phosphate (6, 31, 42). In addition, several OM proteins are accessible on the outer leaflet of the *Brucella* surface (7, 17) and the OM of the smooth (S) *Brucella* species contains a native hapten polysaccharide possibly intertwined with the O chain of the LPS (1). Some of these features correlate well with the observed permeability of the *Brucella* OM to hydrophobic compounds (25) and with the resistance of these bacteria to EDTA, Tris, some detergents (32), and oxygen-independent killing mechanisms (3, 35). Despite the fact that all of these OM features have been presented as key factors in the intracellular survival of *Brucella* sp. (6, 26, 35), it has been difficult to dissociate the relative contribution of each type of surface molecule in these events.

A general procedure employed in functional studies of *Brucella* OM molecules has been to test the properties of isolated compounds (10, 26, 34, 35). Although this approach has contributed to the understanding of the function of certain surface molecules, the data obtained do not necessarily correlate with

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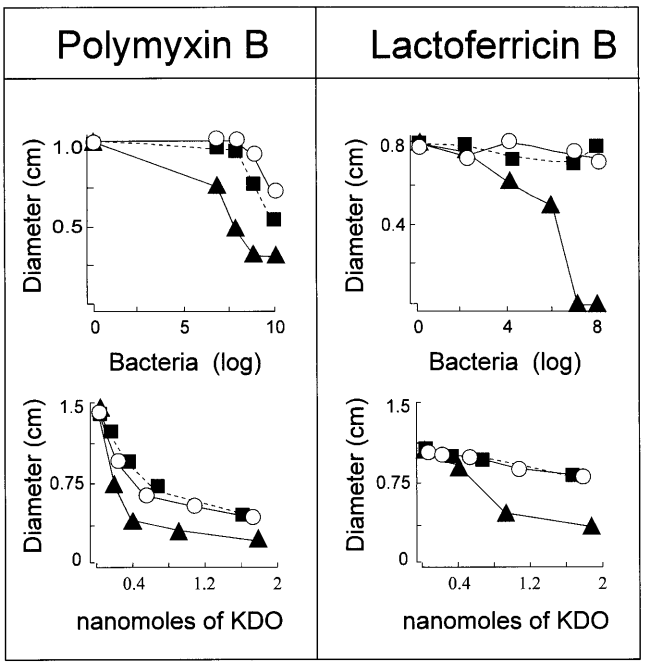

FIG. 1. Binding of polymyxin B and lactoferricin B to bacterial cells and LPS micelles. ○, S *B. abortus* S19; ■, R *B. abortus* 45/20; ▲, *S. montevideo* SH94. Binding of polymyxin B or lactoferricin B to bacterial cells (top) or to LPS micelles (bottom) was proportional to the size of the bactericidal halo in agar plates inoculated with *E. coli* cells as the detecting system. The standard deviation at each point was less than 7% of the value. KDO, 3-deoxy-D-manno-octulosonic acid.

the contribution of the same molecules in the intact OM, where interactions with other components are relevant. An alternative strategy which has provided useful information on many gram-negative species, but with very limited results regarding *Brucella* organisms, has been the use of mutants or genetically engineered bacteria showing a detectable dysfunction which could be associated with the presence, absence, or alteration of a particular OM molecule (12, 21, 41). We have recently employed an additional strategy involving the construction of OM bacterial chimeras by incorporating foreign molecules in the OM of *Brucella* mutants (26). In the present study, we combined several of the above approaches to determine the role of LPS in the previously described (26, 32) resistance of *Brucella* cells to polymyxin B, lactoferricin B (two bactericidal peptides with different structures and biological activities), and EDTA, as well as in the characteristic permeability of *Brucella* cells to hydrophobic compounds (25). The results demonstrated that LPS plays a determinant role in all of these properties.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The characteristics and culture conditions of S-type *B. abortus* S19, rough (R)-type *B. abortus* 45/20, *Salmonella montevideo* SH94, and *Escherichia coli* ATCC 29648 have been described previously (26). To carry out sensitivity assays with cationic peptides and EDTA, bacteria were grown on blood agar plates and harvested in the appropriate buffer. The bacteria were suspended in 1% neopeptone (Difco Laboratories, Detroit, Mich.) in 0.01 M phosphate buffer (pH 7.4) to test the pentacationic lipopeptide polymyxin B sulfate (Sigma Chemical Co., St. Louis, Mo.). Lactoferricin B (a cationic peptide of 25 amino acids, derived from the proteolytic digestion of the iron-binding protein lactoferrin B, kindly provided by W. Bellamy, Morinaga Dairy Company, Higashihara, Japan), was tested on bacteria suspended in 0.1 M phosphate buffer (pH 7.4).

LPS and OM preparations. The extraction and purification of LPS from S *B. abortus* S19 and *S. montevideo* SH94 have been described elsewhere (9, 13). Briefly, *Brucella* LPS was obtained from the phenol phase of the phenol-water extraction method (2, 4). Purification was carried out by digesting the LPS (20 mg of LPS per ml of 5 mM $MgCl_2$–10 mM Tris-HCl [pH 7.5]) in three consecutive steps with 20 mg each of DNase I and RNase A and 50 μg of proteinase K (all from Sigma) for 24 h at 25°C. Following centrifugation at 10,000 × *g*, the preparation was subjected to gel filtration in the presence of chaotropic agents in accordance with previously published work (30). The LPS was then centrifuged at 100,000 × *g* for 6 h at 4°C, and removal of phospholipids and ornithine lipids was obtained with chloroform-methanol-water (1:8:0.8, vol/vol), followed by chloroform–methanol–7 M ammonia (65:25:4, vol/vol). *S. montevideo* LPS was obtained from the aqueous phase of the phenol-water extraction method described by Wesphal and Jann (47). Purification was carried out by resuspension of LPS (20 mg/ml) in 5 mM $MgCl_2$–10 mM Tris-HCl (pH 7.5), sonication, and digestion with DNase I, RNase A, and proteinase K for 24 h at room temperature. This LPS was then reextracted with phenol-water, dialyzed, and recovered by centrifugation at 100,000 × *g* for 6 h at 4°C. All LPS preparations were lyophilized after extensive dialysis and analyzed by standard procedures (9). Data on the chemical and physical characteristics of these LPS preparations have been published previously (13, 20, 31). *B. abortus* OM fractions from R and S strains were prepared and characterized as described above (14).

Antibodies. Polyclonal antibodies against *S. montevideo* SH94 (serotype C1) were produced in mice by intraperitoneal injection of 0.1 ml of an emulsion containing 200 μg of LPS per ml of Freund adjuvant (Sigma) in 0.133 M NaCl–0.01 M phosphate-buffered saline (pH 7.4). A first injection given in complete adjuvant was followed by three injections at 5-day intervals in incomplete adjuvant. The mice were bled 4 days after the last injection, and the serum obtained was tested by indirect enzyme immunoassay (38). Those animals with higher titers were bled, and the sera were stored at −20°C in 50% glycerol until used at a later stage. The production and characteristics of monoclonal antibodies against the *Brucella* LPS C/Y epitope (Baps-C/Y) and the R *Brucella* LPS R1 epitope (Baro-1) have been described previously (38). Goat anti-mouse immunoglobulin conjugated with gold particles of 5 and 10 nm were purchased from Amersham International. Rabbit anti-mouse immunoglobulin-fluorescein conjugates were obtained from Sigma. All antibodies were tested for specificity against the required LPS epitopes by enzyme immunoassay (38) and by immunoelectron microscopy as explained below.

Construction of *B. abortus*-LPS chimeras and *S. montevideo-B. abortus* LPS micelle hybrids. Coating of live R *B. abortus* 45/20 with heterologous LPS was carried out as described before (26), with modifications. Twenty milligrams of *S. montevideo* or *B. abortus* S19 LPS per milliliter of 0.01 M phosphate buffer (pH 7.4) was sonicated for 20 s at maximum frequency (Branson Sonifer 450; Branson Ultrasonics Corp., Danbury, Conn.) and sterilized by filtration (0.2-μm pore size; Millipore Corp., Bedford, Mass.) before being used. Two hundred microliters of fresh *B. abortus* 45/20 cells, harvested in 1% peptone in 0.01 M phosphate buffer (pH 7.4) and adjusted to 4×10^7 CFU/ml, was mixed with 200 μl of different concentrations of heterologous LPS. The bacterium-LPS mixtures were sonicated briefly (three 1-s pulses) and incubated for 18 h at 40°C. The unbound LPS was removed by washing the cells repeatedly in 0.01 M phosphate buffer (pH 7.4) at 14,000 × *g* for 15 min at 25°C. The bacterial pellets were resuspended in 200 μl of the appropriate buffer and used immediately for sensitivity and immunochemical assays.

LPS micelle hybrids were produced by the procedure described by Rudbach et al. (39). Briefly, 1 mg of S-type *B. abortus* S19 LPS was mixed with 1 mg of *S. montevideo* LPS in 2 ml of double-distilled water and the mixture was sonicated for 30 s at maximum frequency. Following this, 2 ml of 2% Na-deoxycholate (Sigma) in 0.1 M Tris-HCl (pH 8.5) was added to the LPS mixture, which was incubated for 15 min at 20°C. The preparation was then precipitated with 6 volumes of ethanol at −20°C for 18 h, centrifuged at 10,000 × *g*, washed with 6 volumes of cold ethanol, and resuspended in water at a final concentration of 400 μg/ml. Finally, it was extensively dialyzed against double-distilled water. Controls were treated the same way as the mixture, except that the micelles were formed with homologous LPSs. *B. abortus* S-LPS micelles have a rodlike shape and are 0.1 to 0.05 μm long, whereas the *Salmonella* and *Salmonella-Brucella* LPS micelle hybrids have a rodlike shape and are 0.25 to 0.2 μm long (23, 29, 36).

Sensitivity to polymyxin B, lactoferricin B, and EDTA. Bacterial sensitivity to the cationic peptides and EDTA was measured as the percentage of cell viability after exposure to the agent. Sterile EDTA (5 mM) and stock solutions of polymyxin B sulfate (2 mg/ml) and lactoferricin B (2 mg/ml) prepared in double-distilled water were maintained at −20°C for later use. For polymyxin B, the assays were carried out in 1% peptone in 0.01 M phosphate buffer (pH 7.4). For lactoferricin B, the assays were performed in 0.1 M phosphate buffer (pH 7.4) because this peptide precipitated in the presence of peptone. Two hundred microliters of the *B. abortus*-LPS chimeras in the appropriate buffer were divided into two aliquots of 4×10^6 CFU in 100 μl: one was used as a control, whereas the other was supplemented with the above cationic agents for sensitivity assays. Native bacteria were harvested from fresh agar plates and adjusted to 4×10^7 CFU/ml. One hundred microliters (4×10^6 CFU) was incubated for 20 min (for polymyxin B) or 180 min (for lactoferricin B or EDTA) at 37°C with different concentrations of EDTA or the cationic peptides to be tested; the live bacteria were then counted on tryptic soy agar (Difco) plates. Experiments were run in quadruplicate, and the results were expressed as the percentage of CFU (mean ±

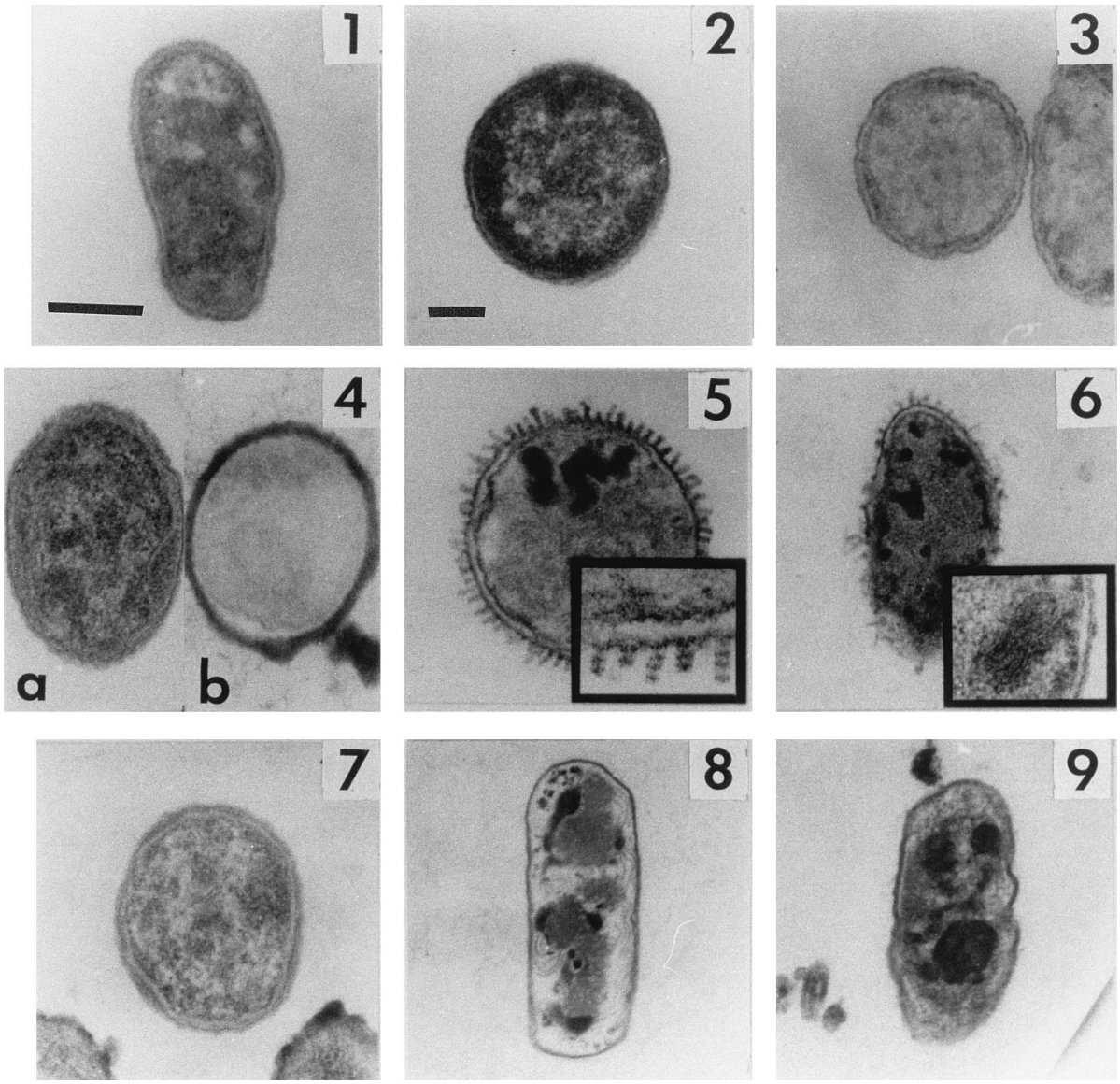

FIG. 2. Transmission electron microscopy of polymyxin B- and lactoferricin B-treated R *B. abortus* 45/20 (panels 1, 4, and 7), *S. montevideo* SH94 (panels 2, 5, and 8), and *B. abortus-S. montevideo* LPS chimeras (panels 3, 6, and 9). Panels: 1 to 3, untreated controls; 4a, 5, and 6, bacteria treated with 100 U of polymyxin B; 4b, bacteria treated with 15,000 U of polymyxin; 7 to 9, bacteria treated with 10 μg of lactoferricin B. The bar in panel 1 (0.25 μm) corresponds to native and chimeric *B. abortus* cells. The bar in frame 2 (0.25 μm) corresponds to *S. montevideo* cells. The inserts in panels 5 and 6 show augmented details (magnification, ×150,000) of rolling of the inner membrane and OM blebbing.

standard deviation) with respect to the controls. The Student *t* test and variance analysis were performed for statistical analysis.

Binding of cationic peptides to bacterial cell OM and LPS. For all binding experiments, bacterial suspensions were adjusted at different concentrations in 0.01 M phosphate buffer (pH 7.4), mixed with 10 μl containing 100 U of polymyxin B or 40 μg of lactoferricin B, and incubated at 37°C for 20 and 180 min, respectively. For LPS and OM binding experiments, preparations were resuspended at different concentrations in 0.1 M phosphate buffer (pH 7.4) and 100-μl aliquots were mixed with 10 μl containing 100 U of polymyxin B or 40 μg of lactoferricin B and incubated at 37°C for 20 and 180 min, respectively. Following incubation, all of the mixtures were centrifuged at 14,000 × *g* for 20 min and the supernatants were tested for microbicidal activity as follows: 5-μl volumes of the supernatants were dispensed into 3-mm wells punched in 1% peptone–1% glucose–0.8% yeast extract–1% agar plates previously inoculated with 10^8 CFU of *E. coli* ATCC 29648 per ml. After incubation of the plates for 12 h at 37°C, the amount of each unbound cationic peptide was estimated by measuring the diameter of the inhibition halo by using the pure peptide as the standard.

Fluorimetry. The fluorescent probe *N*-phenyl-1-naphthylamine (NPN) (Sigma) has been used in OM permeability studies because its quantum yield increases when it is transferred from a hydrophilic to a hydrophobic environment (24, 40, 43). Fluorimetric assays were carried out as described by Martínez de Tejada and Moriyón (25), with some modifications. Exponentially growing native bacterial cells or *B. abortus*-LPS chimeras were resuspended in 1 mM KCl–10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.2) at an optical density at 600 nm of 0.1 and transferred to fluorimetric cuvettes. Fluorescence was monitored at 20°C with an LS-50 fluorimeter (Perkin-Elmer Ltd., Beaconsfield, England) set as follows: excitation wavelength, 350 nm; emission wavelength, 420 nm; slit width, 5.0 nm. The results were expressed in relative fluorescence units.

Transmission electron microscopy procedures. For electron microscopy experiments, native bacteria and *B. abortus*-LPS chimeras were adjusted to 4×10^9 to 4×10^{10} CFU/ml. Proportionately, the number of bacteria and peptide concentrations were maintained as described above for the sensitivity assays. Samples were fixed with 4% glutaraldehyde (Sigma) in 0.1 M phosphate buffer (pH 7.4) for 1 h at 4°C, pre-embedded in 3% low-gelling-temperature agarose (Sea Plaque; FMC Corp.), and stored for 12 h in fixative solution at 4°C. Agarose pellets were cut into 3-mm^3 pieces, immersed in fixative solution for 2 h at 25°C, washed three times for 10 min each with phosphate buffer (pH 7.4), and postfixed

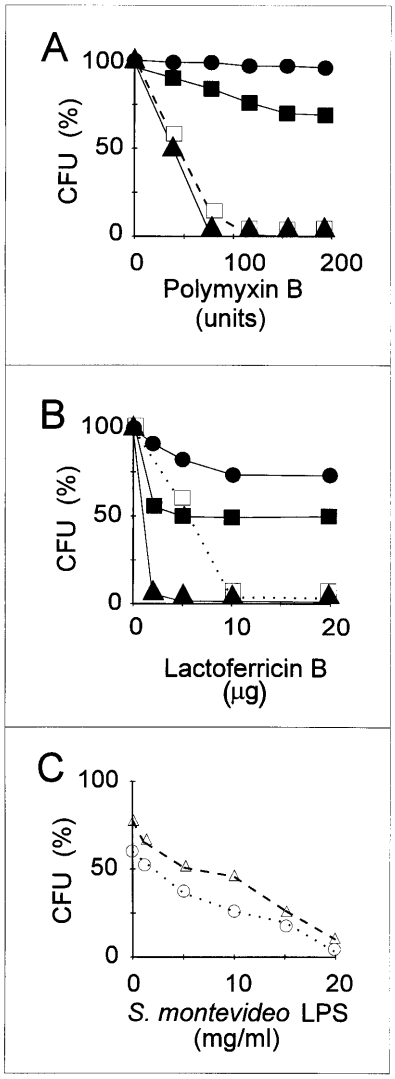

FIG. 3. Bactericidal dose-response curves of cationic peptides and their action on *B. abortus-S. montevideo* LPS chimeras coated with different concentrations of LPS. Symbols: ●, *B. abortus* S19; ■, R *B. abortus* 45/20; ▲, *S. montevideo* SH94; □, *B. abortus-S. montevideo* LPS chimeras; ○, polymyxin B; △, lactoferricin B. The standard deviation at all points was less than 10% of the value.

with 1% osmium tetroxide (Agar Scientific Ltd., Cambridge, England) in 0.1 M phosphate buffer (pH 7.4) for 2 h at 25°C (37). The fixed agarose sections were washed again three times under the conditions described above, dehydrated in increasing concentrations of ethanol and propylene oxide (Agar Scientific Ltd.), and embedded in Spurr resin (Agar Scientific Ltd.) as described elsewhere (37). Polymerization was performed at 60°C for 48 h, and ultrathin sections were obtained with a Sorvall MT2 ultramicrotome. The sections were placed on 5% collodion-coated 100-mesh grids. The sections were stained with 4% uranyl acetate (Agar Scientific Ltd.) for 15 min (37) and then with 2.66% lead citrate (Agar Scientific Ltd.) for 15 min. The samples were observed and photographed with a Hitachi 1100 transmission electron microscope (Hitachi Scientific Instruments, Mountain View, Calif.) operating at 100 kV.

Immunochemical procedures. The partition of heterologous LPS into the OM of R *B. abortus* 45/20 was demonstrated by indirect immunogold staining. Fresh native or chimeric bacterial cells were fixed with 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 1 h and washed three times at 14,000 × *g* for 20 min, and the pellets were resuspended in 50 μl of 0.133 M NaCl–0.01 M phosphate buffer (pH 7.4) containing 5% normal goat serum (Dako A/S). Gold grids (400 mesh) covered with Formvar-carbon film (Agar Scientific Ltd.) were covered with 25 μl of a bacterial suspension and incubated at 25°C for 30 min. The excess liquid was removed with filter paper, and the grids were washed with 0.133 M NaCl–0.01 M phosphate buffer (pH 7.4) containing 0.1% normal goat serum (Dako). Incubation with the appropriate concentration of monoclonal or polyclonal antibodies diluted in 0.133 M NaCl–0.01 M phosphate buffer (pH 7.4) containing 0.1% normal goat serum was carried out at 25°C for 30 min. The grids were washed five times with 0.133 M NaCl–0.01 M phosphate buffer (pH 7.4) containing 0.1% normal goat serum and incubated for 30 min at 25°C with a goat anti-mouse immunoglobulin-gold conjugate diluted in the same buffer. The grids were washed three times with 0.133 M NaCl–0.01 M phosphate buffer (pH 7.4) and three times with double-distilled water, the excess liquid was removed, and the grids were dried at 25°C. Samples were analyzed without further staining and photographed in a Hitachi H1100 transmission electron microscope. Agglutination and immunofluorescence assays of native bacteria and *B. abortus*-LPS chimeras was carried out by standard procedures described elsewhere (38). The relative amount of heterologous LPS adsorbed to the bacterial cells was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by densitometry (13, 44).

RESULTS

Binding of polymyxin B and lactoferricin B to live bacterial cell OMs and LPS. The results of the binding of polymyxin B and lactoferricin B to live bacterial cells and LPS are presented in Fig. 1. *S. montevideo* cells adsorbed more polymyxin B and lactoferricin B than did any of the *B. abortus* strains tested. *B. abortus* 45/20 cells had a slightly higher affinity for polymyxin B than did their S *B. abortus* S19 counterparts. Moreover, none of the *Brucella* strains bound lactoferricin B. LPS fractions of the three bacterial strains had a higher affinity for polymyxin B than for lactoferricin B. *S. montevideo* LPS had a higher affinity than the *B. abortus* LPS preparations. On a dry-weight basis, the R-LPS from *B. abortus* 45/20 bound more polymyxin B than did its S-LPS counterpart (data not shown); however, on the basis of 3-deoxy-D-manno-octulosonic acid content, both *Brucella* LPSs bound similar quantities of peptides. As with the results observed with viable bacterial cells, *Brucella* LPS showed a lower binding capacity for lactoferricin B than for polymyxin B. The isolated OM fractions of the S and R *Brucella* strains bound amounts of peptides similar to those bound by the bacterial cells (data not shown).

Properties of *B. abortus*-LPS chimeras. Compared with the native R *B. abortus* controls, the coating procedure with heterologous LPS had no detectable effect on the growth rate, nor did it cause any decrease in the number of CFU during 8 to 48 h of culture. Transmission electron microscopy of chimeras showed no difference with respect to native *B. abortus* cells (Fig. 2, panel 3). The coating was dose dependent, achieving its maximum at 20 mg of LPS per 4×10^7 CFU/ml (Fig. 3C), and more efficient when the cell-LPS mixture was sonicated prior to incubation. However, the quantity of LPS initially adsorbed by R *B. abortus* 45/20 cells was estimated to be 27 to 31% of the amount of LPS added. This indicates that the coating proceeded under conditions of extreme LPS saturation and that the equilibrium had to be aggressively pushed towards the heterologous LPS for this to be incorporated into the OM of the foreign bacteria. The agglutination and indirect immunofluorescence tests demonstrated that anti-*S. montevideo* LPS antibodies reacted with *B. abortus-S. montevideo* LPS chimeras whereas anti-*B. abortus* S-LPS (anti-C/Y epitope) monoclonal antibodies reacted with *B. abortus* 45/20-*B. abortus* S19 LPS chimeras. Immunofluorescence was observed in almost all of the cells exposed to the coating procedure. Immunogold detection showed a homogeneous and abundant peripheral distribution of the heterologous LPSs on the surface of almost all bacteria (Fig. 4C) which was dispersed over time (8, 24, and 48 h) in a semiconservative manner. This uniform distribution of foreign LPS was similar to that observed for the native S-LPS (Fig. 4A) but different from the patching pattern of R-LPS (Fig. 4B) on the surfaces of S and R *Brucella* cells. The relative amount of foreign LPS on the surface of the chimeras (gold particles in Fig. 4C) was 65 to 75% of the native LPS on the surface of S *B. abortus* cells (gold particles in Fig. 4A). A

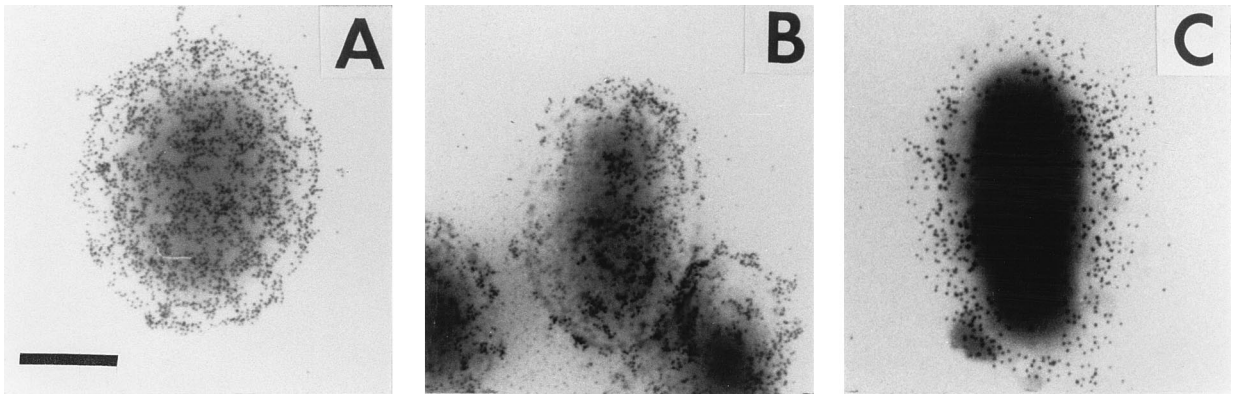

FIG. 4. Immunogold staining of native *B. abortus* and *B. abortus-S. montevideo* LPS chimeras. (A) S *B. abortus* S19 stained with an anti-*B. abortus* S-LPS (C/Y epitope) monoclonal antibody and an anti-mouse immunoglobulin-gold (5 nm) conjugate. (B) R *B. abortus* 45/20 stained with an anti-*B. abortus* R-LPS (R1 epitope) monoclonal antibody and an anti-mouse immunoglobulin-gold (5 nm) conjugate. (C) *B. abortus-S. montevideo* LPS chimeras stained with an anti-*S. montevideo* SH94 LPS antibody and an anti-mouse immunoglobulin-gold (10 nm) conjugate. Bar, 0.25 μm.

monoclonal antibody against R *Brucella* cells (Baro-1) could not access or detect R-LPS epitopes in the OM of S *Brucella* cells or the chimeras. Native R *B. abortus* showed no agglutination or unspecified labelling by either anti-*B. abortus* S-LPS or anti-*S. montevideo* LPS antibodies.

Sensitivity of *B. abortus*-LPS chimeras to polymyxin B, lactoferricin B, and EDTA. In accordance with previous data (26), R *B. abortus* was more sensitive to polymyxin B and lactoferricin B than was S *B. abortus* but considerably more resistant than the enteric bacteria (Fig. 5). Compared with native R *B. abortus* cells, the chimeras were either more resistant or more sensitive to the action of peptides, depending on the LPS used in the coating procedure (Fig. 5). When the heterologous LPS corresponded to the less sensitive S *B. abortus*, the chimeras became significantly ($P < 0.001$) more resistant. On the other hand, when the LPS was from *S. montevideo*, the chimeras were more susceptible than the uncoated controls. Similarly, *B. abortus-S. montevideo* LPS chimeras and *S. montevideo* cells were more sensitive than the native *Brucella* strains to the action of EDTA (Fig. 5). The polymyxin B dose-response curve of the *B. abortus-S. montevideo* LPS chimeras was very similar to that of *S. montevideo*: both became totally inhibited at approximately 100 U of polymyxin B (Fig. 3A). Likewise, at lactoferricin B doses higher than 10 μg, the chimeras were as susceptible as the *S. montevideo* cells. At doses lower than 5 μg, the chimeras behaved the same way as native *B. abortus* 45/20 cells (Fig. 3B). These effects were due to the heterologous *S. montevideo* LPS inserted in the OM of the chimeras, as a direct correlation was observed between the amount of LPS used to coat the R *B. abortus* cells and sensitivity to the peptides (Fig. 3C).

To directly assess the action of polymyxin B and lactoferricin B, ultrathin sections were investigated by transmission electron microscopy (Fig. 2). Treatment of *B. abortus* with a dose of polymyxin B or lactoferricin B lethal for *S. montevideo* did not affect the structure of the cells (Fig. 2, panels 4 and 7). The use of large quantities of polymyxin B resulted in the deposition of an electron-dense layer on the surface of *B. abortus* with no detectable cell damage (Fig. 2, panel 4b). Control experiments showed that insoluble polymyxin B included in agarose stained with uranyl and osmium salts, as with the electron-dense coat observed around polymyxin B-treated *B. abortus*. No differences were observed between S and R *B. abortus* strains (data not shown). In contrast, the exposure of *B. abortus-S. montevideo* LPS chimeras to peptides produced severe cell damage

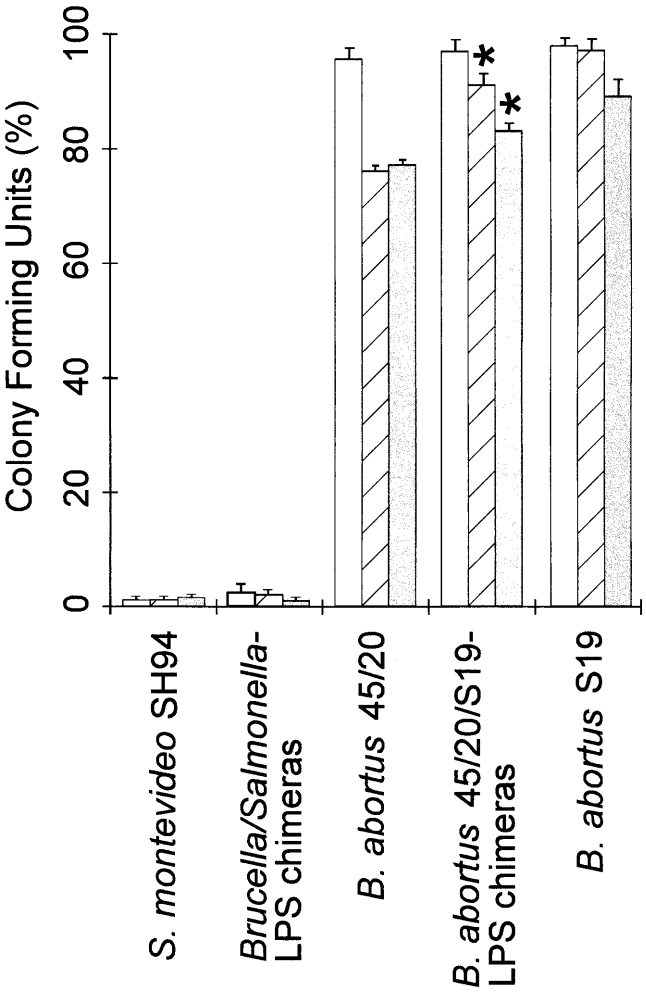

FIG. 5. Sensitivity of native bacteria and *B. abortus*-LPS chimeras to polymyxin B, lactoferricin B, and EDTA. ▨, polymyxin B; ▨, lactoferricin B; □, EDTA. The percent reduction of CFU was calculated with respect to control bacterial suspensions without EDTA or peptides. An asterisk indicates a probability of <0.001 for a difference between *B. abortus*-LPS chimeras and native *B. abortus* 45/20 treated with the peptides.

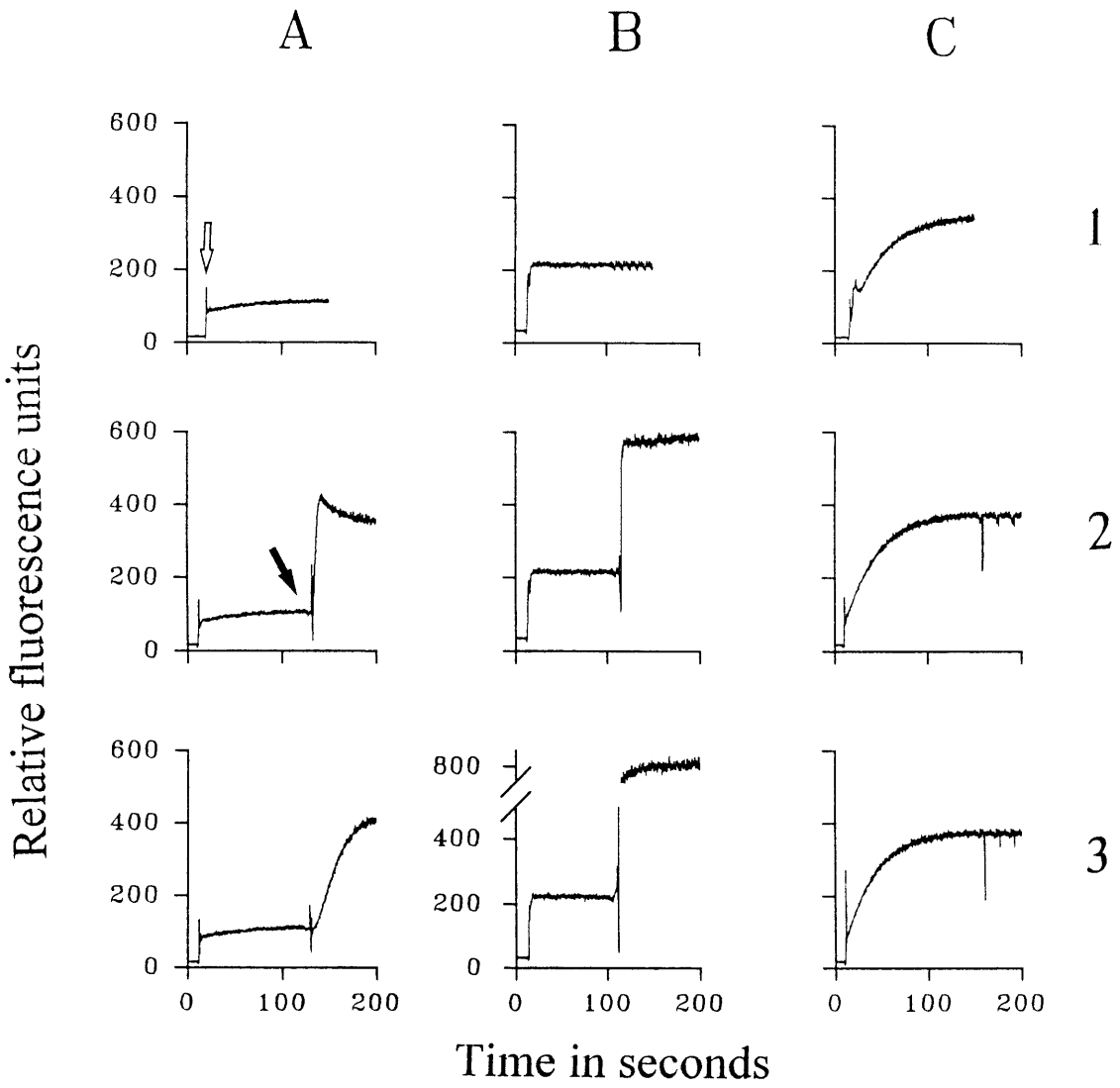

FIG. 6. Fluorimetry assays of bacteria treated with polymyxin B or lactoferricin B. Columns: A, *S. montevideo* SH94 cells; B, *B. abortus-S. montevideo* LPS chimeras; C, *B. abortus* 45/20 cells. Rows: 1, controls after addition of the fluorescent probe NPN; 2 bacteria after addition of NPN followed by polymyxin B; 3, bacteria after addition of NPN followed by lactoferricin B. The arrows indicate the times of addition of NPN (⇨) and the peptide (➔).

which paralleled that observed for *S. montevideo* cells (Fig. 2, panels 6 and 9). The detrimental effect of polymyxin B was characterized by blebbing of the OM, vacuole formation, and rolling of the inner membrane (Fig. 2, panels 5 and 6). Likewise, the damage induced by lactoferricin B was characterized by detachment of the inner membrane, formation of internal electron-dense granules, and vacuolization (Fig. 2, panels 8 and 9). Blistering of the OM was observed only after limited interaction with lactoferricin B (48). These phenomena were conspicuous at higher rather than lower peptide doses (more than 50 U of polymyxin B or more that 10 μg of lactoferricin B), and the bacterial death observed at different peptide concentrations was commensurate with the CFU reductions presented in Fig. 3.

Effects of cationic peptides and EDTA on the partition of NPN into bacterial OMs and LPS micelles. The permeability attributes and the action of cationic peptides and EDTA on *B. abortus-S. montevideo* LPS chimeras were compared to those of *S. montevideo* and *B. abortus* cells by studying the partition of NPN into the OMs (Fig. 6). In the absence of the above agents, only a limited amount of NPN partitioned into the OM of *S. montevideo*, as shown by the small increase in fluorescence measured after addition of NPN (Fig. 6A1). On exposure of *S. montevideo* cells to polymyxin B or lactoferricin B, a rapid increase in the entry of NPN was detected (Fig. 6A2 and A3). Different NPN uptake kinetics were observed for the two bactericidal peptides: the NPN uptake caused by polymyxin B was faster and steeper than that caused by lactoferricin B. In contrast to these observations, NPN partitioned spontaneously into native *B. abortus* OMs (Fig. 6C1) and the kinetics of NPN uptake were not altered by either polymyxin B (Fig. 6C2) or lactoferricin B (Fig. 6C3). As with *S. montevideo*, only limited amounts of NPN were partitioned into the OM of the *B. abortus-S. montevideo* LPS chimeras (Fig. 6B1) and peptide exposure suddenly increased the NPN uptake (Fig. 6B2 and B3).

The above experiments were repeated with S-LPS micelles instead of live cells (Fig. 7). *S. montevideo* LPS was less permeable to NPN than was the S LPS from *B. abortus* (28 versus 54 relative fluorescence units after NPN addition, respective-

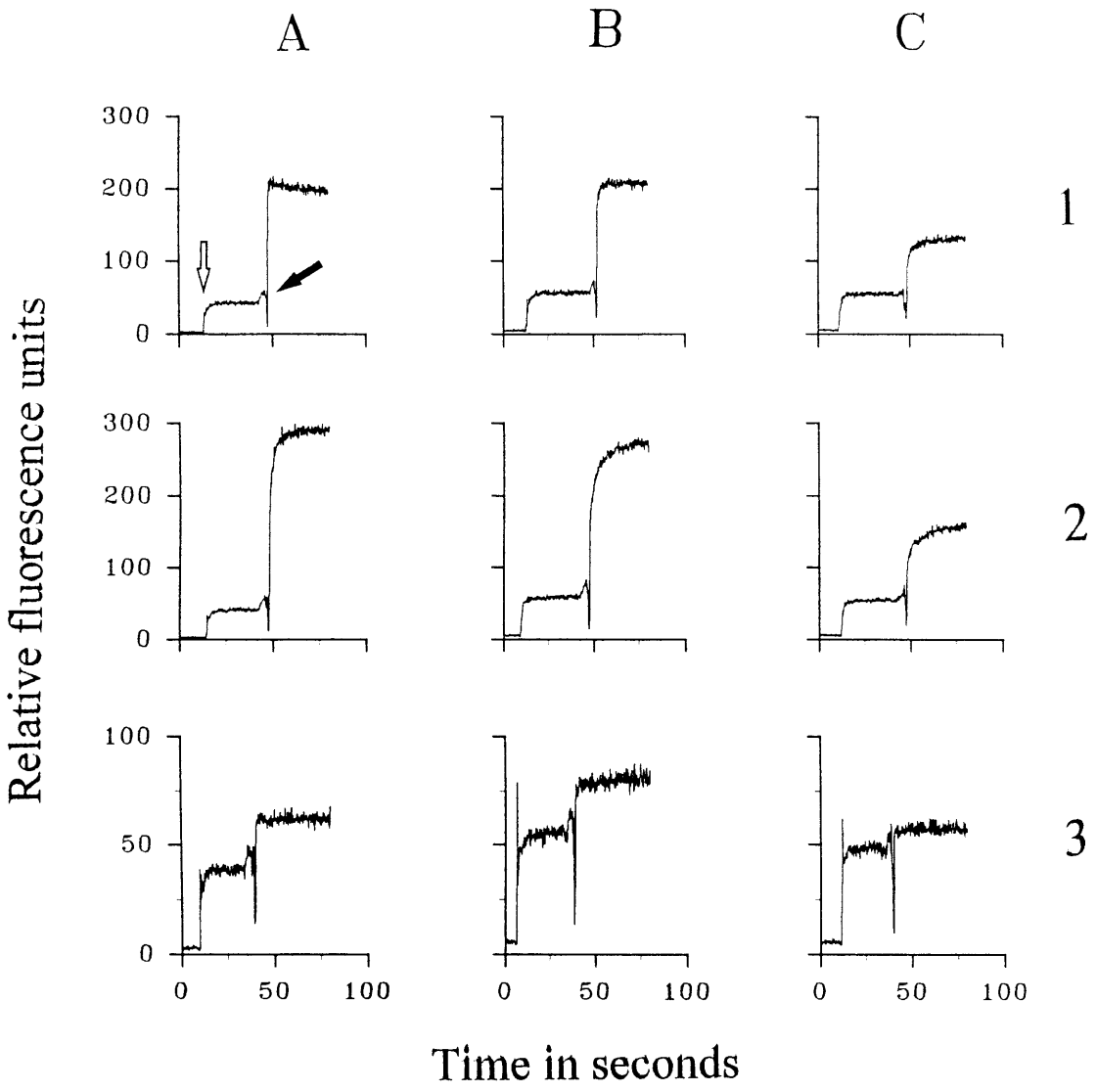

FIG. 7. Fluorimetry assays of hybrid LPS micelles with polymyxin B, lactoferricin B, or EDTA. Columns: A, *S. montevideo* SH94-LPS micelles; B, *S. montevideo-B. abortus* LPS micelles; C, *B. abortus* S19-LPS micelles. Rows: 1, LPS micelles after addition of the fluorescent probe NPN followed by polymyxin B; 2, LPS micelles after addition of NPN followed by lactoferricin B; 3, LPS micelles after addition of NPN followed by EDTA. The arrows and the corresponding change in relative fluorescence indicate the times of addition of NPN (⇨) and the corresponding peptide or EDTA (➔).

ly). Although the S-LPS micelles of *B. abortus* were sensitive to polymyxin B (Fig. 7C1) and lactoferricin B (Fig. 7C2), both peptides had a greater effect on the LPS micelles of *S. montevideo* (Fig. 7A1 and A2). Moreover, the latter LPS was sensitive to EDTA (Fig. 7A3) while that of *B. abortus* was comparatively resistant (Fig. 7C3). In a manner consistent with the observations made regarding native bacteria and the *B. abortus-S. montevideo* LPS chimeras, hybridization of *B. abortus* S LPS with *S. montevideo* LPS produced micelles that showed polymyxin B, lactoferricin B, and EDTA sensitivities similar to those of *S. montevideo* LPS (Fig. 7B1, B2, and B3). It is worth noting that the relative NPN uptake of the hybrid micelles was similar to that of *B. abortus* S LPS (approximately 50 relative fluorescence units after NPN addition in both cases).

DISCUSSION

On the basis of the present and previous works (25, 26, 35), we propose that LPS is the main cause of the resistance of *Brucella* spp. to cationic molecules and EDTA and to the increased permeability to hydrophobic substances. The principal support for this is the sensitivity and permeability properties of the *B. abortus* LPS chimeras, which followed those of the LPS donor bacteria. Moreover, the susceptibility to cationic peptides was proportional to the amount of heterologous LPS inserted into the OM. In *Salmonella* spp., the action of EDTA and cationic substances responds to a very different mechanism (11, 18, 46). Chelating agents compete for the Ca^{2+} or Mg^{2+} used to stabilize the OM by salt bridging adjacent LPS molecules together. The absence of metal ions destabilizes the OM, resulting in LPS release and disintegration of the cell envelope. Cationic peptides can mimic the metal ions, displace metals, and insert themselves into the bilayer. Because these molecules are much bulkier than metal ions, the OM is destabilized, resulting in cell death. Under this perspective, the resistance of *Brucella* cells to EDTA is consistent with the reduced divalent-cation stabilization of LPS in the OM of these species (32).

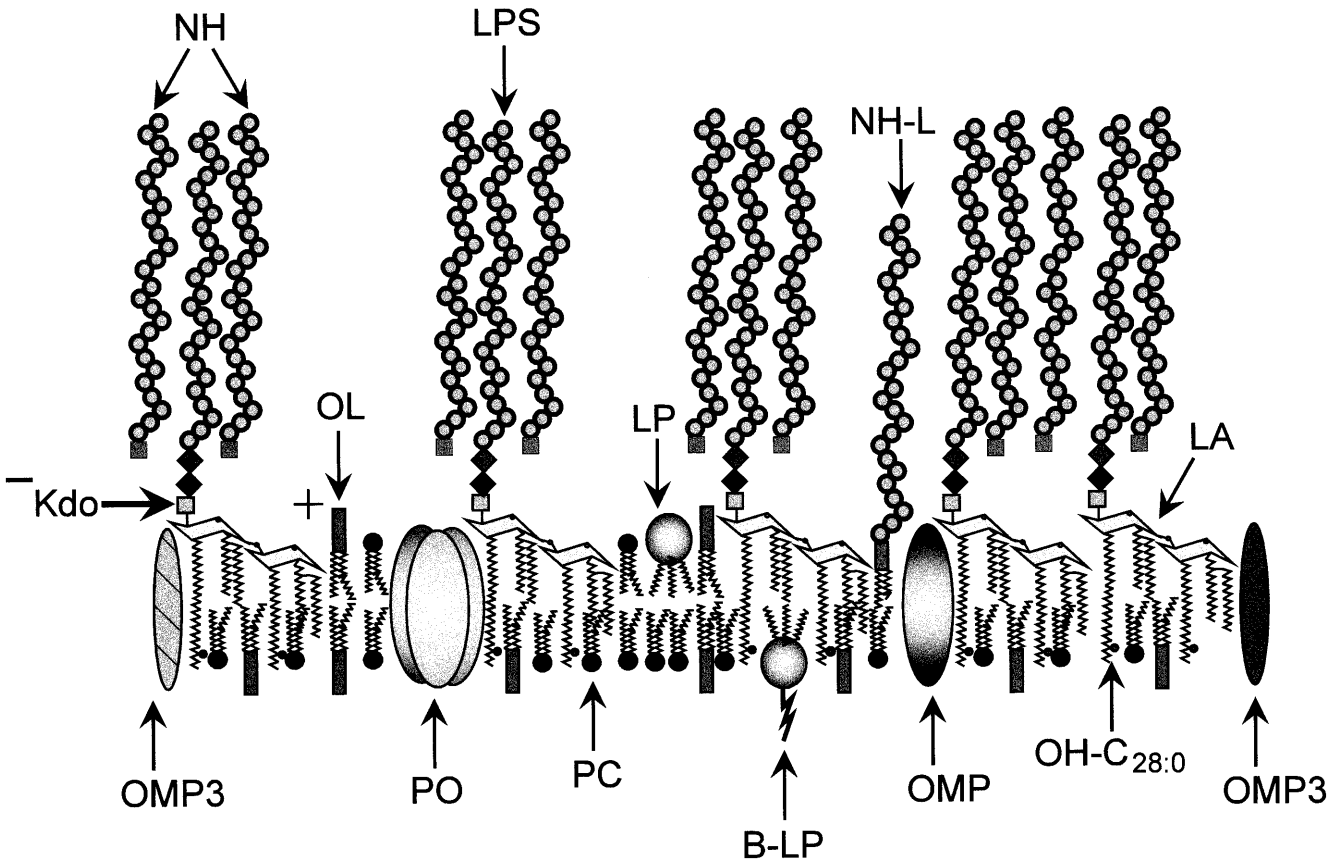

FIG. 8. Schematic representation of S *Brucella* sp. OM. Abbreviations: Kdo, 3-deoxy-D-manno-octulosonic acid; LA, lipid A; LP, free lipoprotein; B-LP, bound lipoprotein; NH, native hapten polysaccharide; NH-L, lipid-bound native hapten polysaccharide; PC, phosphatidylcholine; OH-$C_{28:0}$ hydroxylated $C_{28:0}$ fatty acid; OL, ornithine lipids; OMP, OM proteins; OMP3, OM protein group 3; PO, porin.

Likewise, the comparatively low number of negatively charged groups in the *Brucella* LPS (31, 32) may account for the reduced affinity of the *Brucella* OM for cationic peptides such as polymyxin B and lactoferricin B. To this we must add the relative contribution of the O-chain and OM-associated native hapten polysaccharides to the resistance to cationic peptides, as suggested by the difference in susceptibility between R and S *Brucella* strains and the increased resistance of *B. abortus–B. abortus* S19-LPS chimeras in comparison with R *B. abortus*. It is worth noting that the resistance of *Brucella* cells was more conspicuous when the S-type LPS was integrated into its native OM. This was demonstrated by the fact that *Brucella* S-LPS micelles were partially permeabilized by the action of both peptides while *Brucella* cells were not.

The cellular injuries produced by polymyxin B and lactoferricin B in several proteobacteria have been described in detail (5, 15, 22, 45, 46, 48). In the first instance, polymyxin B establishes an ionic linkage between the peptide amino groups and the negatively charged groups in the lipid A and core region of the LPS (18). The second step occurs when polymyxin B inserts its aliphatic chain into the OM, producing morphological and permeability changes which destroy the bacteria. Although the initial steps of lactoferricin B binding to the OM also include ionic interactions with the LPS, the physical and permeability changes differ from those induced by polymxyin B. The fact that the *B. abortus-S. montevideo* LPS chimeras showed morphological alterations similar to those of *S. montevideo* supports the notion that the events proceed according to the model proposed for enterobacterial cells (18, 45, 48); the *S. montevideo* LPS negatively charged groups provided the initial target for these cationic molecules, and disorganization proceeded in accordance with the characteristics of the peptide and the LPS type rather than with the properties of the non-LPS OM components. Thus, our experiments reinforce the idea that the *Brucella* LPS is in itself an efficient shield against polycations. Other *Brucella* OM molecules, such as phosphatidylcholine and ornithine lipids, whose micelles are not easily disrupted by polymyxin B (11, 19), either are limited to reinforcing this property or possess other functions. Similarly, *Brucella* OM proteins which seem to be similar to those of other proteobacteria (33, 34) cannot be excluded, although their direct participation is unlikely.

The electron microscopy observations showed differences in the peptides' bactericidal performance which suggest that the resistance of *Brucella* cells to polycations works on two different levels. Firstly, the initial ionic interaction is either abrogated (lactoferricin B) or greatly reduced (polymyxin B), depending on the peptide. Secondly, the bactericidal effect is also hampered beyond this action, as shown by the intense polymyxin B deposition on the *B. abortus* OM which did not lead to cell damage (Fig. 2, panel 4b). An alternative explanation is that the hydrophobic, and not the cationic, domain of polymyxin B is that which interacts most with the *Brucella* OM. In both cases, the resistance of *Brucella* cells to polymyxin B should be related to the hydrophobic portion of the LPS. This is consistent with the resistance displayed by the *Brucella* cell

envelope to the action of detergents (32). Furthermore, the mechanism by which the LPS chimeras are generated should reflect this same property since it represents the insertion without cell damage of a foreign amphiphilic molecule.

It is feasible that the increased hydrophobicity of *Brucella* LPS could favor the entry of NPN through surface areas covered by LPS and that some phospholipids dispersed among LPS molecules in the OM could create spots suitable for the partition of hydrophobic permeants (25). The observations that the permeability of the *B. abortus-S. montevideo* LPS chimeras to NPN was lower than that of *B. abortus* and showed a sudden entry of the probe similar to that of *S. montevideo* cells coincide with these hypotheses. Those effects should be expected if the proposed phospholipid patches become substituted with newly inserted foreign LPS and if a mixed R *B. abortus-S. montevideo* LPS lattice is assembled on the OM of the chimera. Moreover, the profile of permeability to the NPN probe demonstrated by *B. abortus* LPS micelles differed from that displayed by *Brucella* organisms and was similar to that shown by *Salmonella* or *Salmonella-Brucella* LPS micelle hybrids, reinforcing the notion that non-LPS OM molecules participate in the arrangement of the LPS lattice.

It is not known whether the heterologous LPS in the OMs coexist with or displaces native LPS and if synthesis of the latter could be inhibited by the presence of the former. It can be concluded from the LPS hybrid experiments and research carried out on mixed micelles of LPS and phospholipids or on interactions between LPS and heterologous OM proteins that *Brucella* LPS can coexist with foreign molecules in vitro in stable membrane arrangements (this work; 33). The fact that antibodies did not detect native R-LPS on the surface of the chimeras and the discovery that the heterologous LPS displayed a homogeneous and abundant distribution on these cells support the proposal that the foreign LPS molecules were tightly packed in the outer leaflet of the chimeric OM.

How the *Brucella* LPS lattice is stabilized in the OM is not known. A model of the S *Brucella* OM, based on the present and previous works (1, 6, 7, 25–34, 42), which is commensurate with our results and working hypotheses is presented in Fig. 8. It is feasible to propose that the *Brucella* LPS lattice is stabilized in the OM by means of the long (up to 30 carbon atoms) fatty acids of the lipid A moieties which may establish strong hydrophobic interactions among them. It may also be that the hydroxyl group linked at the end of the 28 to 30-carbon fatty acids of the lipid A span the OM and stabilize the LPS by exposing this polar head to the inner leaflet. Another possibility is that few anionic groups of the LPS are shielded by cationic ornithine lipids. Finally, it may be that strong interactions on the surface of the OM between the O polysaccharide chain and the native hapten polysaccharide contribute to stabilization of the LPS in this layer.

Although several factors have been implicated in the virulence of *Brucella* organisms, the means by which these bacteria withstand the inimical environment within phagocytes have not been elucidated. In contrast to many other pathogens, *Brucella* organisms do not produce exotoxins, cytolytic enzymes, fimbriae, flagella, and capsules or harbor plasmids, and no antigenic variation has been found (27). Since most of the humoral defenses are directed against the LPS, and R variants are, in general, less pathogenic than their S counterparts, attention has been centered on the LPS molecule (16, 28, 35, 41). However, the emphasis has often been focused on the "endotoxic" action of *Brucella* LPS (41) rather than on its structure and interaction with other OM compounds. We have demonstrated that the LPS constitutes a primary obstacle for bactericidal cationic substances. Therefore, our hypothesis is that the *Brucella* LPS is a virulent factor solely because it creates an efficient protective barrier for withstanding the digestive activities of phagocytes and because it lacks some of the most conspicuous biological properties of the "classical" LPS leading to the activation of phagocytes, a strategy that may be repeated by other intracellular α proteobacteria (27, 31).

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