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Lysosomal Accumulation and Recycling of Lipopolysaccharide to the Cell Surface of Murine Macrophages, an In Vitro and In Vivo Study

Claire Forestier,* Edgardo Moreno,† Javier Pizarro-Cerda,* and Jean-Pierre Gorvel2*

In this study, we detailed in a time-dependent manner the trafficking, the recycling, and the structural fate of Brucella abortus LPS in murine peritoneal macrophages by immunofluorescence, ELISA, and biochemical analyses. The intracellular pathway of B. abortus LPS, a nonclassical endotoxin, was investigated both in vivo after LPS injection in the peritoneal cavity of mice and in vitro after LPS incubation with macrophages. We also followed LPS trafficking after injection of macrophages with B. abortus strain 19. After binding to the cell surface and internalization, Brucella LPS is routed from early endosomes to lysosomes with unusual slow kinetics. It accumulates there for at least 24 h. Later, LPS leaves lysosomes and reaches the macrophage cell surface. This recycling pathway is also observed for LPS released by Brucella S19 following in vitro infection. Indeed, by 72 h postinfection, bacteria are degraded by macrophages and LPS is located inside lysosomes dispersed at the cell periphery. From 72 h onward, LPS is gradually detected at the plasma membrane. In each case, the LPS present at the cell surface is found in large clusters with the O-chain facing the extracellular medium. Both the antigenicity and heterogeneity of the O-chain moiety are preserved during the intracellular trafficking. We demonstrate that LPS is not cleared by macrophages either in vitro or in vivo after 3 mo, exposing its immunogenic moiety toward the extracellular medium. The Journal of Immunology, 1999, 162: 6784–6791.

Lipopolysaccharide, the most abundant component of the Gram-negative bacteria outer membrane, is the main Ab-inducing Ag during bacterial infections and one of the most biologically active molecules (1). LPS released in the body fluids by live or killed bacteria in the form of blebs or membrane vesicles (2) is readily ingested by professional phagocytes following pinocytosis, receptor-mediated endocytosis, macropinocytosis, or phagocytosis (3–7). Alternatively, phagocytosed Gram-negative bacteria may release LPS within the intracellular milieu (8, 9). Enterobacterial LPS has been detected in phagosomes, endosomes, the Golgi complex, the nucleus, mitochondria, and cytoplasm (6, 10–12). It has been shown that during the first 2 days after ingestion, LPS is mainly found in the form of small, loosely packed, vacuoles that later seem to coalesce into larger and tightly packed compartments (13). However, the characterization of the LPS-containing compartments during its intracellular routing in a time-dependent manner has not as yet been determined.

Due to their accumulation within macrophages, LPSs have been regarded as slowly processable molecules (14–16). Macrophages are able to deacylate and dephosphorylate the enterobacterial lipid A moiety (17–19), which is eventually exocytosed to the extracellular medium (20). Other investigations have suggested that the O-chain and the core oligosaccharide of enterobacterial LPS are also degraded within macrophages (13, 20) by mechanisms that have not yet been elucidated. The slow processing of LPS within phagocytic cells may be due to the absence of a molecular machinery capable of efficiently digesting it. Alternatively, LPS may directly alter the constitutive intracellular trafficking of molecules within cells perhaps in conjunction with its toxic effect. In this respect, the elucidation of the trafficking of LPS and the identification of LPS-containing compartments are necessary to understand the pathophysiological effects mediated by this molecule and to resolve the fate and the intracellular processing of nonprotein Ags. This last aspect is relevant in the light of investigations, which have clearly demonstrated that microbial glycolipids may be presented to T cells in the context of nonclassical histocompatibility Ags (21, 22).

In this work, we detailed in a time-dependent manner the intracellular trafficking of the low endotoxic Brucella abortus LPS (23) in murine peritoneal macrophages. We studied the fate of LPS after in vitro incubation and in vivo i.p. injection of purified LPS, and after Brucella infection in vitro. The results show that LPS follows the endocytic pathway described for proteins Ags, but with unusually slow kinetics. We observed that Brucella LPS initially accumulates within lysosomal compartments, followed by a gradual recycling to the plasma membrane, where it arranges as large clusters. In contrast to enterobacterial LPS, no alteration in the immunochemical properties of Brucella LPS was observed in spite of the slow trafficking within lysosomes, suggesting that this molecule resists intracellular degradation mechanisms.

Materials and Methods

Mice and cells

Eight- to fifteen-week-old C3H/HeN female mice (Jackson ImmunoResearch, West Grove, PA) were used in all experiments. Peritoneal murine macrophages were obtained after cervical dislocation from normal mice or from mice injected i.p. with 0.4 ml of B. abortus S19 LPS (1 mg/ml). Each
killed mouse received an i.p. injection of 20 ml of sterile PBS/10% sucrose at 15°C. After injection, the abdomen of animal was massaged and the liquid was extracted from the peritoneal cavity. Pooled fluids were centrifuged at 12000 rpm for 10 min at 4°C and the pellets were resuspended in DMEM (Life Technologies, Cergy-Pontoise, France) supplemented with 10% FCS, 2 mM glutamine, 10 mM sodium pyruvate, 10 mM nonessential amino acids, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies). A total of 2 × 10⁵ peritoneal cells was plated on 12-mm glass coverslips in 24-well tissue culture plastic dishes, 10⁵ cells/ml in 96-well plates (Costar, Cambridge, MA) or at 10⁴ cells/35-mm dish (Nuncl, Poly Labo) for immunofluorescence, ELISA, or immunoblotting analysis, respectively. After incubation for 1 h at 37°C under 5% CO₂, nonadherent cells were removed from the wells by aspiration, and the adherent macrophages were rinsed twice with PBS and incubated with fresh culture medium.

**Bacteria and LPS preparations**

The characteristics of smooth type *B. abortus* S19 (Professional Biological, Denver, CO) have been previously described (24). Bacteria were grown at 37°C in tryptic soy broth (TSB; Difco, Detroit, MI) to stationary phase, and aliquots were frozen at −70°C in TSB-glycerol 30%. A log-phase culture bacteria was prepared by incubating a thawed aliquot (5 × 10⁶ CFU/ml) in TSB for 15–48 h at 37°C under agitation. Bacterial numbers were determined by comparing the OD at 600 nm with a standard curve.

The extraction and purification of LPS from smooth and rough *B. abortus* have been described elsewhere (25, 26). Briefly, smooth *Brucella* LPS was obtained from the phenolic phase of phenol-water extraction method (27). Purification of LPS (20 mg/ml LPS in 5 mM MgCl₂/10 mM Tris-HCl, pH 7.5) was conducted by repeated digestion with nucleases and proteinase K (Sigma, St. Louis, MO) for 24 h at 25°C. The LPS was then centrifuged at 100,000 × g for 6 h at 4°C, and the removal of phospholipids and ornithine lipids was achieved with chloroform/methanol/water (1:8:0.8, v/v/v), followed by chloroform/methanol/7 M Ammonia (65:25:4, v/v/v). LPS was then reextracted with phenol/water 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 (26, 28). For internalization studies and injections in mice, LPS was dissolved in water at the appropriate concentration with the aid of sonication and autoclaved before use.

**Cell infection and LPS endocytosis**

After removal of culture medium, macrophages grown on coverslips in 24-well tissue plates were inoculated with 500 μl of a bacterial suspension in culture media at a rate of 50 bacteria/cell. Culture plates were centrifuged for 10 min at 1000 rpm at room temperature, followed by incubation for 20 min at 37°C under 5% CO₂. Cells were washed five times with culture medium to remove nonadherent bacteria, and monolayers were further incubated with culture medium containing 50 μg/ml of gentamicin (Sigma) to kill extracellular brucellae. In long-term experiments, this medium was replaced twice: at 1 h with medium containing 25 μg/ml gentamicin, and at 24 h with medium supplemented with 5 μg/ml gentamicin.

Macrophage layers grown on glass coverslips (for immunofluorescence experiments), 96-well plates (ELISA), or 35-mm plastic dishes (Costar, Cambridge, MA) or at 10⁴ cells/35-mm dish (Nuncl, Poly Labo) for immunofluorescence, ELISA, or immunoblotting analysis, respectively. After incubation for 1 h at 37°C under 5% CO₂, nonadherent cells were removed from the wells by aspiration, and the adherent macrophages were rinsed twice with PBS and incubated with fresh culture medium.

**Antibodies**

Murine mAb against O-chain C/Y epitope (Baps C/Y) was produced and characterized, as previously described (29). Cow polyclonal serum against *B. abortus* S-LPS was described in earlier works (29). Briefly, the serum was collected from a *B. abortus*-infected cow and was characterized to react strongly against two O-chain epitopes (C/Y and A) from S-LPS, core epitopes from isolated R-LPS, and isolated lipid A epitopes. At the dilution used, this serum does not react against core or lipid A when LPS molecule is complete (core and lipid A epitopes are hidden due to the presence of O-chain). Rabbit polyclonal antiserum against R-LPS was produced by immunizing rabbits with a purified preparation of *B. abortus* 45/20 R-LPS (30). This antiserum reacts against core epitopes from the R-LPS, but it does not react against the O-chain, core epitope from S-LPS, and lipid A (29). Rabbit IgGs anti-cow Ig and Baps C/Y mAb were linked to horseradish peroxidase, as previously described (31). At the dilution used, none of the polyclonal or mAbs used demonstrated reactivity against LPS-unreated macrophages. Rabbit polyclonal IgGs anti-cation-independent mannos-6-phosphate receptor (anti-Ci-M6PR, Dr. B. Hoflack, Institut Pasteur de Lille, Lille, France); rabbit anti-cathepsin D (Dr. S. Kornfeld, Washington University School of Medicine, St. Louis, MO); and rabbit anti-giantin (Dr. H. P. Hauri, University of Basel, Basel, Switzerland) were used for immunofluorescence experiments. Secondary Abs used were: goat Texas red-conjugated anti-cow IgG and donkey FITC-conjugate anti-rabbit IgG (Jackson ImmunoResearch). Rabbit IgG-peroxidase anti-mouse Ig and goat IgG-peroxidase anti-rabbit IgG conjugates were used (Sigma) as secondary Abs for Western blotting and ELISA.

**Confocal microscopy**

For fluorescent confocal microscopy, macrophages grown in coverslips were fixed at room temperature with 3.7% paraformaldehyde in PBS, pH 7.4, for 20 min, followed by 10-min incubation with 0.1 M glycine to saturate aldehyde groups and with 0.1% saponin for cell permeabilization. Then cells were incubated with a PBS solution containing 0.1% saponin/5% mouse serum/5% horse serum for 20 min to block unspecific binding. Primary Abs diluted in the same buffer were added to the cells for 30 min. After extensive washings with 0.05% saponin in PBS, macrophages were incubated for 30 min with secondary fluorescent Abs. Coverslips with adherent macrophages were washed, mounted in Mowiol ( Hoechst, Frankfurt, Germany), and viewed under a Leica TCS 4D confocal microscope (Leica Lasertechnik, Heidelberg, Germany). In all immunofluorescence experiments, LPS was revealed using the antiserum from infected cow, followed by anti-cow IgG Ab conjugated to fluorescein.

**Western blotting**

After internalization of *B. abortus* LPS, macrophages were lysed in PBS/1% Nonidet P-40 detergent for 30 min, and treated with 1 mg/ml of proteinase K (Sigma) at 37°C for 30 min. Lysates were run in 12% SDS-PAGE and electrophoretically transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked in PBS/5% milk/0.05% Tween-20 (Sigma) for 2 h. Primary and secondary Abs were successively added in this buffer, each being left for 2 h before staining with the enhanced chemoluminescence system (ECL; Amersham, Courtaboeuf, France). As primary anti-LPS Abs, Baps C/Y, cow, and rabbit antiserum were used to detect the different epitopes of LPS in macrophage cell lysates.

**Dot-blot analysis**

Peritoneal liquids from LPS-inoculated mice were recovered by injecting 5 ml of PBS/10% sucrose in peritoneal cavity; the liquid was extracted and centrifuged to eliminate macrophages. Supernatants were filtered through a 0.45-μm cutoff membrane (Gelman Sciences, Ann Arbor, MI) to eliminate cellular debris. A total of 100 μl of supernatant was applied to Immobilon-P membrane (Millipore) in a dot-blot manifold (Bio-Rad, Hercules, CA). The membrane was removed from the manifold and blocked with 0.2% BSA for 1 h before adding the peroxidase-conjugated anti-O-chain Ab (Baps C/Y). Detection has been done with the enhanced chemoluminescence system.

**ELISA assays**

After LPS internalization, macrophages were fixed with 3.7% paraformaldehyde in PBS, then incubated with PBS/10% mouse serum/0.1% saponin to block unspecific sites and to permeabilize the cells. Parallel cultures were not permeabilized to measure LPS exclusively on the cell surface. Cells were then incubated with 100 μl of the antiserum from infected cow diluted in PBS/5% normal mouse serum, with or without the addition of saponin for nonpermeabilized cells. Membranes were incubated with 100 μl of horseradish peroxidase-conjugated anti-bovine Ig was added for 45 min at room temperature. After washings, 100 μl of tetramethylbenzidine substrate (Sigma) was added to the cells. After 30-min incubation, the enzymatic reaction was stopped by adding 10 μl of 0.5 M H₂SO₄, and the color reaction was read at 450 nm OD. To estimate at which LPS dose the OD corresponded, various fixed amounts of LPS were deposited on an Immobilon-P membrane in a dot-blot manifold, as described above. Each spot was cut off, and the membrane was then deposited in distinct wells on
24 well plates. The amount of LPS was revealed by ELISA assays (described above) using peroxidase-conjugated anti-O-chain Ab (Baps C/Y).

**Results**

**LPS is delivered with slow kinetics to lysosomes, where it accumulates**

To define the internalization pathway of *Brucella* LPS in macrophages, we compared, by indirect immunofluorescence, the intracellular distribution of LPS with several markers of endocytic compartments and the Golgi apparatus. Macrophages were initially incubated with LPS for 1 h at 4°C, followed by different periods of chase at 37°C. After 10 min of internalization at 37°C, LPS (A) colocalized with transferrin-FITC (B) and, at 60 min, LPS (C) was no longer associated with transferrin-FITC (D). At 90 min, LPS (E, G) was found in a compartment positive for the CI-M6PR (F) and negative for giantin, a specific marker of the Golgi apparatus (H). Bar = 10 µm.

![FIGURE 1. Intracellular trafficking of *Brucella* LPS in murine peritoneal macrophages. Cells were incubated with 15 µg/ml of LPS in cell culture medium for 1 h at 4°C, then washed and further incubated for various times at 37°C. After paraformaldehyde fixation, macrophages were processed for double immunofluorescence. LPS was detected by indirect immunofluorescence using the antiserum from *Brucella*-infected cow. After 10 min of internalization at 37°C, LPS (A) colocalized with transferrin-FITC (B) and, at 60 min, LPS (C) was no longer associated with transferrin-FITC (D). At 90 min, LPS (E, G) was found in a compartment positive for the CI-M6PR (F) and negative for giantin, a specific marker of the Golgi apparatus (H). Bar = 10 µm.](Image 3)

**FIGURE 2. Transient accumulation of *Brucella* LPS in lysosomes.** In A, macrophages untreated with LPS were stained with the antiserum from a *B. abortus*-infected cow (Aa) and with anti-cathepsin D Ab (Ab). In B, macrophages were incubated with LPS as in Fig. 1. Times of chase were from 1–48 h. LPS, detected with the antiserum from *Brucella*-infected cow, is shown in Ba, Bc, and Be, and cathepsin D in Bb, Bd, and Bf. At 5 h at 37°C, LPS (Ba) reached lysosomes positive for cathepsin D (Bb) and accumulated in this compartment even after 24 h (Bc and Bd). At 48 h, LPS (Be) was mainly found outside lysosomes (Bf). Bar = 10 µm.

The persistence of LPS in macrophages prompted us to investigate the fate of LPS at late time points. At 48 h, LPS was still present in macrophages. Although a minor proportion of LPS-containing vesicles remained positive for the lysosomal marker cathepsin D, the majority of LPS was found within intracellular nonlysosomal compartments (Fig. 2, Be and Bf) and at the plasma membrane forming large clusters (Fig. 3B). This result suggests that, after lysosomal accumulation, LPS escaped lysosomes and migrated to the cell surface. We also estimated the relative amounts of cell surface-bound versus intracellular LPS by ELISA (Fig. 3A). Even...
though the majority of LPS was rapidly internalized after 10 min, as shown in Fig. 1, A and B, after 2 h, 25% of total LPS still resided at the plasma membrane (Fig. 3A). Complete internalization occurred by 24 h. Then LPS gradually reached the macrophage cell surface after 2 days, LPS aggregates forming large clusters, distributed inside and outside lysosomal compartments as well as at the plasma membrane (Figs. 4 and 3D). This unique cellular distribution pattern persisted up to 90 days postinjection (not shown). Peritoneal macrophages from LPS-injected mice, harvested at 60 days postinjection, were then cultured in vitro in the absence of LPS. Fig. 3C shows that LPS started to recycle from lysosomal and nonlysosomal compartments to the plasma membrane after 48 h. The majority of LPS molecules reached the macrophage cell surface after 5 days (Fig. 3C), confirming the results shown in Fig. 3A.

The immunochemical properties of Brucella LPS are unaltered during its intracellular trafficking

As shown in Fig. 5, A and B, native Brucella S-LPS displays a characteristic m.w. heterogeneity related to the different lengths of the O-chain, core oligosaccharide, and lipid A (35), whereas R-LPS appears as a lower m.w. band (Fig. 5C). After internalization and trafficking within macrophages, LPS did not show any significant SDS-PAGE profile variation or altered antigenic reactivity against a collection of monoclonal anti-O-chain Abs (Fig. 5A) as well as polyclonal Abs from a B. abortus-infected cow, and was shown to react strongly against two O-chain epitopes (C/Y and A) from S-LPS, core epitopes from isolated R-LPS, and isolated lipid A epitopes (Fig. 5B), suggesting that LPS was not cleaved during its intracellular trafficking in peritoneal macrophages. In addition, we never detected the appearance of R-LPS (Fig. 5C), indicating that the O-chain moiety was not cleaved from S-LPS. By comparing the signal intensity corresponding to cell-associated LPS with that of purified LPS, the amount of cell-associated LPS was estimated to 1 ng/10^6 cells in agreement with ELISA measurements.

Intracellular LPS released from digested bacteria is detected at the plasma membrane

We have shown that the attenuated B. abortus S19 strain is degraded within lysosomes of phagocytes (36). To explore the fate of LPS released intracellularly after the degradation of phagocytosed attenuated S19 bacteria in lysosomes, we followed the intracellular...
 trafficking of S19 Brucella LPS within macrophages at various times postinfection. Forty-eight hours after inoculation, most intact bacteria were found to be within compartments positive for the lysosomal marker cathepsin D (Fig. 6A). At 72 h, most bacteria were degraded within lysosomes, and bacterial debris containing LPS were found within cathepsin D-positive vesicles, scattered throughout the cytoplasm (Fig. 6A), indicating that while bacteria underwent enzymatic degradation in the cathepsin D-positive compartment, the epitopic structure of LPS resisted the intracellular degradation processes. From 72 h onward, LPS was gradually detected at the macrophage plasma membrane (Fig. 6B). Finally, at 6 days postinfection, almost all infected cells exposed high levels of LPS at their plasma membrane (Fig. 6B), indicating that a considerable proportion of glycolipids released by the degraded bacteria followed the recycling pathway described above.

Discussion

Macrophages are specialized cells for capturing, processing, and presenting exogenous Ags to T lymphocytes. They are also the principal target for many intracellular Gram-negative bacteria and their endotoxins. The biological action of LPS on macrophages has been studied extensively (37). Among the less well-characterized events following LPS interaction with macrophages are the intracellular trafficking and processing of internalized LPS. A comprehension of these steps is critical for the understanding of the biological activity and immunogenicity of this nonpeptidic Ag.

In this study, we demonstrate that, in conditions that favor receptor-mediated endocytosis in vitro (38), LPS is rapidly internalized in early endosomes and transported, with slow kinetics, to late endosomes and then to lysosomes, where it accumulates. Later, LPS is found in cathepsin D-negative compartments before it reaches the cell surface, where it forms large clusters. In contrast to other reports, we did not detect Brucella LPS either in the Golgi apparatus, nucleus, mitochondria, nor free in the cytoplasm (6, 10). This discrepancy may be explained by the different experimental procedures, including the use of different target cells and high doses of cytotoxic LPS. Despite the fact that Brucella LPS induces many biological effects, characteristics of so-called typical LPS molecules, it possesses very low toxicity (23), which favors long-term studies in cultured cells and in animals (39). In general, previous studies were performed by using continuous enterobacterial LPS incubation at 37°C, which may allow receptor-mediated uptake, macrophagocytosis, and phase-endocytosis (6, 10). Recent studies from Kitchens et al. demonstrated that the route and kinetics of LPS trafficking depend upon its internalization pathway (CD14 dependent or independent) and its aggregation state, respectively (40, 41), thus offering an explanation for the divergence of the results obtained between different studies.

It has been proposed that the kinetics of intracellular trafficking of different substances is independent of the nature of the ingested molecule in many respects (42–44). However, several studies have demonstrated that LPS follows the endocytic pathway with slow kinetics (11) (Beatty et al., submitted). In agreement with these, we observed a substantial delay in the intracellular transit of LPS from early endosomes to lysosomes, in comparison with proteins such as BSA-FITC, which is rapidly transported from early endosomes to lysosomes within 60 min after internalization and remains located in perinuclear lysosomes until its degradation (45). The slow kinetics of Brucella LPS traffic considerably differs from Brucella organisms. In the case of virulent bacteria, it is known that bacteria evade lysosomes and replicate within the endoplasmic reticulum. For nonvirulent Brucella, the bacteria are found within phagosomes that rapidly fuse with lysosomes, where degradation occurs 12 h postinfection (36). In the latter case, Brucella LPS is consequently released from the outer membrane of the killed bacteria and remains within lysosomes for at least 140 h. This last observation is in agreement with several investigations that have demonstrated the appearance and retention of free LPS within host cells after phagocytosis of different bacteria (8, 9, 13, 46). It is then possible that LPS remains in a nonfunctional lysosomal compartment in which resident proteins, such as cathepsin D and LAMP (lysosome-associated membrane protein), have been degraded or removed. In favor of this is the finding that indigestible polystyrene particles are contained within nonfunctional lysosomes characterized by the absence of hydrolases (44). In addition, it may be that LPS prevents the acquisition of newly synthesized hydrolases arriving from Golgi-derived vesicles, the consequence of which would be the generation of hydrolase-defective lysosomes (47). Therefore, the retention process and coalescence of LPS in large vacuolar aggregates within macrophages seem to be a property of this complex molecule and independent of the bacteria from which it originates.

The clustering of LPS molecules and the formation of discrete patches on the macrophage plasma membrane are consistent with
Bacteria. In association to bacteria or LPS liberated from degraded infected cow serum, which detects either the LPS postinfection times (48, 72, and 120 h). Monitor the presence of surface-bound LPS at different beled with both anti-cathepsin D Ab and B. abortus riphages were permeabilized with saponin (5, 49). Based on recent experiments conducted in our laboratory in which the membrane fluidity of Brucella LPS-treated cells has been measured (unpublished results), and experiments demonstrating the direct (50, 51) or LPS binding protein (LBP)-mediated (52, 53) intercalation of LPS into bilayers, we propose that Brucella LPS is inserted into the cell membranes by its lipid A moiety, exposing its immunogenic O-chain moiety toward the extracellular medium. The existence and the fate of these LPS large clusters in the plasma membrane remain unknown. Although it was found at the cell surface, no free Brucella LPS was detected in supernatants of cell cultures, as demonstrated by the ELISA and colocalization experiments, respectively, suggesting that there is no exocytosis of LPS from macrophages. The fact that LPS disappeared from peritoneal fluids of mice only 3 wk after its injection suggests that phagocytosis of high doses of LPS by peritoneal cells may be the limiting factor for its clearance in vivo. The presence of conspicuous large aggregates in peritoneal macrophages after 6 and 60 days highlights the remarkable stability of this molecule.

After storage in lysosomes, LPS escapes these compartments and appears on the plasma membrane of macrophages, in agreement with the results previously obtained by Korn et al. (54). Although the mechanism by which LPS recycles from intracellular compartments to the plasma membrane remains to be characterized, it is likely that LPS-containing vesicles ensure the transport of LPS from intracellular compartments to the plasma membrane by specific target mechanisms. It may be that the insertion of the lipid A into the membrane of the LPS-containing compartment recruits constitutive recycling molecules, promoting its own transport to the cell surface. Recycling pathways have been partially characterized for complex membrane lipids or exogenous protein Ags. For instance, ceramide-containing sphingolipids have been shown to be translocated from the Golgi apparatus to the plasma membrane by a vesicle-mediated process (55–57). Despite the fact that LPS possesses structural and functional similarities with sphingolipids such as ganglosides and cerebrosides (58, 59), LPS does not recycle by the same mechanism since we never detected this molecule in the Golgi apparatus. It has also been demonstrated that processed proteic Ags recycle from late endocytic compartments to the cell surface by transiting within specialized MHC class II (MHC-II)-enriched compartments, where peptides associate with Ag-presenting molecules (60). It is possible that Brucella LPS, which is also found in late endocytic compartments, recruits and modifies MHC-II products within lysosomes, as suggested by previous experiments in which this molecule was found to interact and induce the formation of SDS-resistant forms (C forms) of MHC-II proteins in B lymphocytes (61) (Forestier et al., submitted). The coalescence of LPS in discrete patches at the plasma membrane may be the result of a combination of the intrinsic properties of LPS, which promotes its own arrangement as crystal layers in the outer leaflet of membranes (62), and the transport of LPS to specific plasma membrane domains by carrier molecules.

Macrophages are capable of processing enterobacterial LPS by a slow mechanism that may involve a combination of oxidative and enzymatic hydrolytic procedures (18–20). These cells modify the chemical structure of any of the three enterobacterial LPS moieties (lipid A, core, and O-chain) and, as a consequence, alter the electrophoretic mobility of this molecule and its reactivity against specific Abs (13). For instance, decylation and dephosphorylation of LPS result in reduced amounts of lipid A fatty acids, less binding of SDS, and slower migration in SDS-PAGE (13, 46, 63). Oxidation and hydrolysis of core sugars diminish the immunoreactivity against core determinants and result in free lipid A detected with specific Abs (13, 35, 64). Commonly, the resulted free O-chain is unable to migrate in SDS-PAGE due to the absence of SDS binding sites (28, 35). Removal of sugar substituents or repeating units from the O-chain causes faster aberrant migration in SDS-PAGE and reduced reactivity to Abs (13, 65). In spite of its...
long residence inside lysosomes, *Brucella* LPS seems to remain unsealed. This observation is based on the unaltered migration pattern in SDS-PAGE of the *Brucella* LPS extractable from macrophages, its consistent reactivity against monoclonal and polyclonal Abs, and the absence of detectable free core or lipid A determinants. The resistance of *Brucella* LPS to macrophage processing may simply reflect the absence of a cellular machinery capable of oxidizing and hydrolyzing this complex molecule. Indeed, the O-chain, the core oligosaccharide, and the lipid A disaccharide backbone of *Brucella* LPS are respectively more resistant to oxidation, acid hydrolysis (35, 66), and deacylation (26) than enterobacterial LPS. In conclusion, the unique chemical structure of *Brucella* LPS may not be prone to regular processing mechanisms described in the case of enterobacterial LPS.

Finally, our results indicate that LPS is retained in an immunologically detectable form in murine peritoneal macrophages for long periods of time, then recycles and associates as clusters at the plasma membrane without detectable modification. The long permanence of LPS within cells of individuals may result at least in several pathologic effects, such as reactive arthritis or hypersensitivity reactions, developed after acute and chronic bacterial infections (67–69). The presence of LPS at the plasma membrane of APC may lead to the activation of T lymphocytes, as it has been shown for other glycolipids (70–75). The recycling phenomenon may explain how LPS activates some T lymphocytes, as recently shown by others (75–78).

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**References**


