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Release of Lipopolysaccharide from Intracellular Compartments Containing *Salmonella typhimurium* to Vesicles of the Host Epithelial Cell

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The biological effects of bacterial lipopolysaccharide (LPS) on eucaryotic cells have traditionally been characterized following extracellular challenge of LPS on susceptible cells. In this study, we report the capacity of *Salmonella typhimurium* to release LPS once it is located in the intracellular environment of cultured epithelial cells. LPS is liberated from vacuolar compartments, where intracellular bacteria reside, to vesicles present in the host cell cytosol. The vesicle-associated LPS is detected in infected cells from the time when invading bacteria enter the host cell. Release of LPS is restricted to *S. typhimurium*-infected cells, with no LPS observed in neighboring uninfected cells, suggesting that dissemination of LPS occurs entirely within the intracellular environment of the infected cell. The amount of LPS present in host vesicles reaches a maximum when intracellular *S. typhimurium* cells start to proliferate, a time at which the entire host cell cytosol is filled with numerous vesicles containing LPS. All these data support the concept that intracellular bacterial pathogens might signal the host cell from intracellular locations by releasing bioactive bacterial components such as LPS.

Bacterial lipopolysaccharide (LPS) is a complex amphipathic molecule located in the outer leaflet of the outer membrane of gram-negative bacteria. The smooth-type LPS consists of an O side chain, a core oligosaccharide, and a lipid-containing disaccharide, termed lipid A, inserted in the membrane (23, 24). The O side chain is formed by the sequential addition of polysaccharides units containing four or five sugars. A given LPS molecule may have from 1- to 100-unit repeats. The heterogeneity in the number of O side chain repeats is the basis of the typical laddering pattern of smooth LPS in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (21, 30). The rough (R)-type LPS lacks the O side chain. Genetic studies have shown that different operons are involved in LPS biosynthesis (reviewed in reference 30). Beside these studies, a large effort has focused on the elucidation of the biological effects of LPS on eucaryotic cells (23, 24). LPS is the main factor responsible for triggering acute activation stages of the host immune system in severe gram-negative infections (23, 24). These activation events evolve to local or generalized inflammation, humoral immune responses, and septic shock. The molecular basis for these LPS-induced abnormalities includes the secretion of cytokines and other second messengers when exogenous LPS interacts with susceptible cells such as leukocytes and macrophages (23, 24, 37).

Despite the large number of reports describing responses of the mammalian cell to LPS recognition (reviewed in reference 24), there is no consensus regarding how LPS is able to activate eucaryotic cells. A partial understanding of the mechanism of action of LPS is starting to emerge, as recent studies have shown the capacity of LPS to trigger signal transduction events, including tyrosine phosphorylation (35, 36), activation of kinases of the Src and Raf pathways (25), and alteration in the level of eucaryotic transcriptional regulators as Egr-1 (2). Although diverse soluble proteins and membrane-bound receptors have been shown to bind LPS (37), the key surface and/or intracellular host target with which LPS interacts to trigger activation of the eucaryotic cell remains to be defined.

Notably, most of what is currently known about biological effects of LPS is based on in vitro studies involving exogenous challenge of susceptible cells with purified LPS components (26). However, experimental evidence has shown that drugs such as taxol and colchicine, which bind exclusively to the intracellular eucaryotic component tubulin, mimic some of the biological effects produced by LPS in eucaryotic cells (4). Included among these effects are the down-regulation of tumor necrosis factor alpha receptors and increased release of tumor necrosis factor alpha in macrophages (4). Indeed, several in vitro studies have shown a direct interaction of LPS with β-tubulin and microtubule-associated proteins (5). Furthermore, it has been shown that LPS rapidly gains access to the cytoplasm of macrophages (18). All this evidence suggests that LPS may trigger some of its biological effects once is targeted to a defined intracellular location.

Many severe infections caused by intracellular bacterial pathogens exhibit pathophysiological effects related to LPS action. Pathogens of this type include members of the genera *Salmonella*, *Shigella*, *Chlamydia*, *Bordetella*, *Neisseria*, *Haemophilus*, *Legionella*, and *Brucella*. These pathogens can reside in the intracellular eucaryotic environment, and it is conceivable that they may use their LPS molecules to signal the host cell from intracellular locations. This assumption is supported by the capacity of certain intracellular pathogens to secrete pathogen-derived components, such as proteins and lipids, to different subcellular compartments of the host eucaryotic cell. Such examples include the release of lipoarabinomannan by *Mycobacterium tuberculosis* to vesicles dispersed in the host cytosol

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(38); the incorporation of a chlamydial protein in the membrane of inclusion bodies within epithelial cells (27); the presence of LPS in symbiosome membranes of amoebae containing X bacteria (19); the release of a pathogen-derived enzyme, such as glutamine synthetase, to the intravacuolar space of phagosomes containing *M. tuberculosis* (14); and the incorporation of parasite-derived components in host intracellular locations apart from parasitophorous vacuoles containing *Toxoplasma gondii* (1) or *Plasmodium falciparum* (7, 34).

Considering these observations, we were interested in determining whether an intracellular bacterial pathogen can also actively release LPS within the host eucaryotic cell. To address this issue, we performed a microscopic analysis of the LPS distribution in *Salmonella typhimurium*-infected epithelial cells. Our results show that LPS is released in large amounts by intracellular *S. typhimurium* to host cell vesicles during the infection. This phenomenon might have considerable implications for signaling mediated by LPS within the eucaryotic intracellular environment.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* strains used in this study were strain SL1344, which is virulent for mice (16), and two avirulent strains, 12-23, a previously described intracellular replication-defective mutant (22), and LV386, an *rfc* mutant capable of producing LPS with only a single O side chain (3). These strains were grown overnight in LB medium at 37°C under static conditions without shaking, prior to infection of epithelial cells.

Epithelial cells. HeLa epithelial cells (human epithelioid carcinoma cells; ATCC CCL2) and Madin-Darby canine kidney (MDCK) cells were grown in Dulbecco's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and maintained as described previously (9).

Bacterial infection of epithelial cells. HeLa and MDCK epithelial cells were grown overnight at 37°C under 5% CO_2 in MEM–10% FBS to 80% confluency in the monolayer (5×10^4 to 8×10^4 cells seeded per well). Cells were washed with phosphate-buffered saline (PBS) (pH 7.4), and fresh MEM–5% FBS medium was added. Bacteria were added to epithelial cells at a ratio of 10:1, and the mixture was incubated at 37°C under a 5% CO_2 atmosphere for 30 min (HeLa cells) or 60 min (MDCK cells). Infected cells were washed three times with PBS and incubated in fresh MEM–5% FBS containing 100 μg of gentamicin per ml. At 2 h postinfection, the medium was replaced by MEM–5% FBS containing a lower concentration of gentamicin (10 μg/ml).

Antibodies and immunofluorescence microscopy. Two different antibodies were used to recognize *S. typhimurium* LPS: rabbit polyclonal anti-*S. typhimurium* LPS (Difco Laboratories, Detroit, Mich.; no. 2948-47-6), and Sal4, a mouse monoclonal immunoglobulin A (IgA) anti-LPS O antigen (31) (kindly provided by M. Neutra, Harvard Medical School, Boston, Mass., and J. M. Slauch, University of Illinois, Champaign, Ill.). To recognize human lysosomal membrane glycoproteins, H4B4, a mouse monoclonal IgG anti-human-h-lamp2 (Developmental Studies Hybridoma Bank, Iowa State University, Ames, Iowa) was used. The secondary antibodies used were Texas red–goat anti-mouse IgG, fluorescein isothiocyanate (FITC)–goat anti-rabbit IgG, and FITC–goat anti-mouse IgA (Jackson ImmunoResearch Laboratories Inc., Bio/Can Scientific, Mississagua, Ontario, Canada). At different times postinfection, epithelial cells were fixed with 3% paraformaldehyde for 20 min at room temperature, and after two washes with PBS, the cells were incubated serially at room temperature with appropriate dilutions of primary and secondary antibodies in a PBS–10% goat serum–0.2% saponin solution (60 min each). The cells were washed three more times with PBS, and coverslips were mounted on glass slides with a drop of mounting medium (Sigma, St. Louis, Mo.). Indirect immunofluorescence was performed using a Zeiss Axioskop microscope under oil immersion.

Electron microscopy. Infected epithelial cells were processed for electron microscopy immunogold labeling by two methods: (i) the progressive temperature decrease method and low-temperature HM20 Lowicryl embedding, and (ii) rapid sample freezing and cryosectioning.

For the progressive temperature decrease method, infected cells were washed twice with PBS and fixed in situ with 3% paraformaldehyde–0.1% glutaraldehyde in PBS for 1 h at 4°C. After extensive washing with PBS, the cells were incubated for 20 min with 0.2 M NH_4Cl to block free aldehyde groups. After two washes with PBS, the cells were dehydrated serially with 30% ethanol (30 min at 0°C), 50% ethanol (60 min at −20°C), and 70, 95, and 100% ethanol (60 min each at −50°C). Lowicryl HM20 embedding was performed as recommended by the manufacturer (J.B. EM Services, Quebec, Canada). Briefly, the cells were infiltrated with a mixture of resin and ethanol (1:1 [60 min] and 2:1 [60 min]) and with pure resin (two incubations, one for 60 min and the second overnight). The entire embedding procedure was done at −50°C. Polymerization of resin was done at −50°C under UV light. Thin sections (0.1 μm) of the samples were collected on gold grids covered with Formvar and carbon and processed for immunogold labeling. Polyclonal rabbit anti-*S. typhimurium* LPS (dilution, 1:200) was used as the primary antibody, and goat anti-rabbit IgG conjugated to 5-nm gold particles (dilution, 1:20; Janssen-CedarLane, Hornby, Ontario, Canada) was used as the second antibody.

Immunogold labeling on thin cryosections was used to increase the efficiency of labelling with the mouse monoclonal antibody H4B4 anti-human-h-lamp2. Infected HeLa epithelial cells were fixed with 3% paraformaldehyde in PBS (pH 7.4) for 60 min at room temperature. After three washes with PBS, the cells were scraped, pelleted (3,000 × *g* for 10 min), and embedded in 10% gelatin by mild centrifugation (3,000 × *g* for 10 min at room temperature). The gelatin was allowed to solidify for 1 h at 4°C. The cell pellet embedded in a block of solidified gelatin was further infiltrated in serial solutions of 0.1, 1, and 2.3 M sucrose in PBS at 4°C (60-min, 60-min, and overnight incubations, respectively). The blocks were trimmed, frozen by being plunged into liquid nitrogen, and sectioned at −90°C with an ultramicrotome (Ultracut E; Reichert) equipped with an FC4 cryochamber. Ultrathin sections (0.1 μm) were transferred to carbon-coated Formvar grids and blocked for 15 min with 10% goat serum in PBS. Antibody labelling was performed by incubating cryosections for 60 min with primary antibodies (rabbit polyclonal anti-LPS [1:200] and mouse monoclonal H4B4 anti-h-lamp2 [1:10]) in a 10% goat serum–PBS solution. After several washes in PBS, the grids were incubated for 30 min with secondary antibodies (goat anti-rabbit IgG conjugated to 5-nm gold particles and goat anti-mouse IgG conjugated to 10-nm gold particles, both at a 1:20 dilution [Janssen-CedarLane]) in 10% goat serum–PBS solution. After being washed, cryosections were coated with a 2% methylcellulose–0.3% uranyl acetate solution and observed in a JEM-1010 electron microscope.

Immunodetection of purified LPS. LPS for electrophoretic analysis was prepared from *S. typhimurium* strains by the method of Hitchcock and Brown (15) and resolved with a Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis system (21, 29). Resolved LPS was silver stained (15) or transferred to a polyvinylidene trifluoride membrane (pore size, 0.2 μm; Trans-Blot; Bio-Rad) with a Miniprotean 2 transfer apparatus (Bio-Rad), and 25 mM Tris–190 mM glycine–20% (vol/vol) methanol as the transfer buffer. Transfer was accomplished at 100 V for 20 min. Immunoblotting was performed with rabbit polyclonal anti-*S. typhimurium* LPS and the mouse monoclonal IgA Sal4 anti-LPS O antigen (31), and the blots were developed as described previously (33).

RESULTS

LPS is present in vesicles of *S. typhimurium*-infected epithelial cells. To address whether intracellular *S. typhimurium* can release LPS within the host eucaryotic cell, we analyzed by indirect immunofluorescence microscopy the distribution of LPS in HeLa epithelial cells infected with the virulent strain SL1344. Labeling with polyclonal rabbit anti-*S. typhimurium* LPS showed the presence of numerous vesicles surrounding intracellular bacteria (Fig. 1a, b, e, and f). We were also able to observe the release of LPS to host cell vesicles in HeLa epithelial cells infected with other *S. typhimurium* wild-type strains such as 14028s and SR11 (data not shown). Furthermore, epithelial cells present in the vicinity, but not containing intracellular bacteria, did not show any component recognized by the antiserum (Fig. 1a and b). This observation suggested that LPS present in infected epithelial cells was released by intracellular bacteria. Nevertheless, the LPS observed in host cell vesicles could be derived from LPS present in the extracellular medium and pinocytosed by the infected epithelial cell.

Considering the latter possibility, we first tested whether bacterial internalization was required to detect the presence of vesicles containing LPS. When bacterial internalization, but not attachment, was blocked by pretreatment of HeLa epithelial cells with cytochalasin D, no LPS-containing vesicles were observed in the infected cells by indirect immunofluorescence microscopy (data not shown). However, this result did not exclude that the macropinocytosis and membrane ruffling accompanying *Salmonella* entry into epithelial cells could facilitate the uptake of extracellular LPS. To discard this possibility, we performed two types of experiments. First, purified *S. typhimurium* LPS (List Biological Laboratories, Campbell, Calif.) and human epidermal growth factor were simultaneously added to serum-starved HeLa epithelial cells. Addition of growth factor to starved cells triggers membrane ruffling and

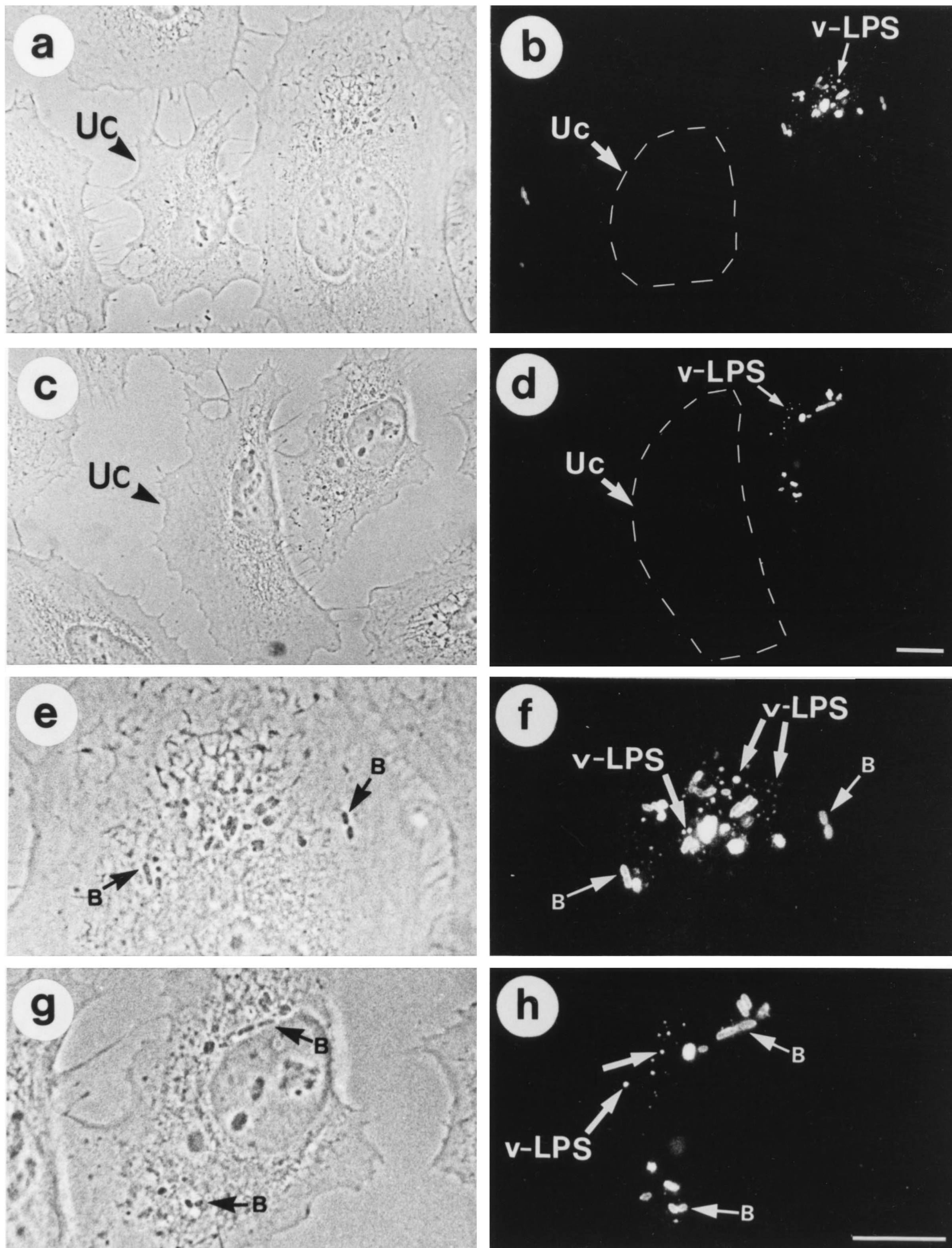

FIG. 1. Appearance of vesicles containing LPS in the cytosol of HeLa epithelial cells infected with *S. typhimurium*. Epithelial cells were fixed at 2 h postinfection and processed for immunofluorescence microscopy with the following primary antibodies: polyclonal rabbit anti-*S. typhimurium* LPS (a and b) or mouse monoclonal IgA anti-O-5 epitope of the *S. typhimurium* LPS (c and d). Infected epithelial cells show vesicles containing LPS (v-LPS) recognized by both types of antibodies. These vesicles appear close to where intracellular bacteria (B) are located. Adjacent uninfected cells (Uc) show no LPS vesicles. (a and c) Phase-contrast images; (b and d) immunofluorescence images of cells shown in panels a and c, respectively; (e and f) high-magnification images of the infected cell shown in panels a and b; (g and h) high-magnification images of the infected cell shown in panels c and d. Bars, 10 μm.

macropinocytosis (10). Upon this treatment, LPS-containing vesicles could not be observed dispersed throughout the host cell cytosol as observed in bacteria-infected cells. This result was consistently reproduced with different amounts of purified LPS, ranging from 200 to 2 ng of LPS per 10^5 HeLa epithelial cells (data not shown). For this experiment, we previously estimated 200 ng of LPS as the total amount of LPS present on the surface of 10^6 bacteria, which is the number of bacteria used to infect 10^5 epithelial cells. Second, we tested whether an FITC-labeled heterologous LPS from *Escherichia coli* O55:B5 (List Biological Laboratories) could be extensively transported to intracellular locations during *S. typhimurium* SL1344 infection of HeLa epithelial cells. Although vesicles containing *S. typhimurium* LPS were detected in all infected cells, only 3% of these cells showed discrete vesicles containing *E. coli* LPS, which were never dispersed throughout the host cell cytosol (data not shown). In addition, FITC-labeled LPS had the same distribution in live cells as in fixed and labeled cells. This excluded the possibility that exogenously added purified LPS was extracted during fixation or immunolabeling (data not shown). Based on these observations, we conclude that the formation of vesicles containing *S. typhimurium* LPS during bacterial infection occurs entirely within the intracellular environment, supporting the concept that *S. typhimurium* located in intracellular compartments probably mediates active liberation of LPS.

The possibility of nonspecific binding of the polyclonal anti-*S. typhimurium* LPS antibodies to structures not related to LPS was discarded by using the mouse monoclonal IgA antibody Sal4, which recognizes the O-5 epitope consisting of acetyl-abequose present in the O side chain of *S. typhimurium* LPS (31). The results obtained when this monoclonal antibody was used to label SL1344-infected HeLa epithelial cells were identical to those obtained with the polyclonal antiserum, and numerous vesicles recognized by this monoclonal antibody appeared exclusively in epithelial cells containing intracellular bacteria (Fig. 1c, d, h, and i).

Release of LPS by intracellular *S. typhimurium* was not restricted to the infection of HeLa epithelial cells. Numerous LPS-containing vesicles were also observed in the cytosol of other cultured epithelial cells, such as MDCK, using both the polyclonal rabbit antibody (Fig. 2a to d) and the mouse monoclonal anti-*S. typhimurium* LPS antibody (Fig. 2e and f).

Additional electron microscopy studies on *S. typhimurium*-infected cells allowed us to confirm the presence of LPS in host vesicles distinct from vesicles containing intracellular bacteria. Immunogold labeling of ultrathin sections of infected HeLa epithelial cells with the polyclonal anti-LPS antibody showed a specific distribution of LPS on intracellular bacteria as well as vesicles having no bacteria (Fig. 3). The result was consistent with the immunofluorescence data, confirming that infected cells have a population of LPS-containing vesicles which do not colocalize with intracellular bacteria.

Release of LPS is enhanced during intracellular bacterial proliferation. To address whether intracellular release of LPS in *S. typhimurium*-infected epithelial cells was related to a defined period of the infection process, we analyzed the amount and distribution of LPS vesicles in infected HeLa epithelial cells fixed at different times following bacterial infection. At early times (30 min) postinfection, LPS vesicles localize to areas surrounding internalized bacteria (Fig. 4a and b). As intracellular bacteria migrate to the perinuclear area of the host cell during the infection, i.e., at 4 h, LPS-containing vesicles become more randomly distributed throughout the host cytosol (Fig. 4d and e). These distribution patterns, showing an apparently restricted location of LPS vesicles, contrast with the numerous LPS vesicles observed in the host cell cytosol at 6 h (Fig. 4g and h), a time at which *S. typhimurium* actively proliferate within epithelial cells (11, 22). Thus, enhanced intracellular release of LPS correlates with an increase in the number of intracellular bacteria.

We have previously shown that *S. typhimurium*-containing vacuoles (SCV) are targeted to compartments containing lysosomal membrane glycoproteins (LGP) of epithelial cells (9, 11), a process that is complete by approximately 2 h postinfection (9). During this period, SCV do not intersect with other organelles of the endocytic route such as late endosomes (9). The analysis of the distribution of LPS vesicles at different times postinfection raised the possibility that LPS vesicles arise by release of LPS contained in the SCV. If this hypothesis is true, vesicles containing both LGP and LPS should be detected. To test this assumption, we performed double immunofluorescence labeling of HeLa epithelial cells infected with *S. typhimurium* SL1344 by using the polyclonal rabbit anti-LPS antibodies and H4B4, a mouse monoclonal antibody recognizing h-lamp2, a human LGP. LPS vesicles and LGP do not colocalize at early times postinfection (30 min; Fig. 4a to c), a time at which SCV have not yet targeted LGP-containing vesicles (9). During this time, colocalization of LPS-containing vesicles with other host early and late endosomal markers was not observed (data not shown). However, at later times (4 h) postinfection, numerous vesicles in the infected cell contained both LPS and LGP (Fig. 4d to f). Colocalization of LPS and LGP was confirmed by cryoelectron microscopy, a technique that provided a higher binding efficiency of the monoclonal anti-LGP antibody. Figure 5 shows single and double immunogold labels of infected HeLa epithelial cells, using anti-LPS and anti-LGP antibodies. LPS was detected in the intravacuolar space of the SCV as well as in the membrane of this compartment (Fig. 5a). Vesicles containing both markers were also visible in locations close to the SCV (Fig. 5c and d) and in the host cell cytosol (Fig. 5e and f).

Notably, at times when intracellular bacteria are actively proliferating (6 h), most of the vesicles containing LPS and filling the host cytosol do not contain LGP. This loss of LGP label from LPS vesicles correlates with an apparent loss of LGP label in compartments containing part of the population of actively growing bacteria (Fig. 4g to i). We have estimated the percentage of intracellular bacteria losing LGP label as approximately 1 to 5% of the total number of intracellular bacteria, in agreement with our previous published observations (9, 11). Included in this estimation are infected cells in which bacteria have not begun to proliferate. Globally, these data indicate an apparent relationship between bacterial growth, loss of LGP label, and extensive intracellular LPS release.

***S. typhimurium* mutants with altered LPS structure are impaired in their ability to release LPS within the host epithelial cell.** The structural requirements for intracellular release of LPS were addressed by utilizing several *S. typhimurium* mutants with deficiencies in LPS biosynthesis. Strain 12-23 is deficient for intracellular replication, is avirulent in the murine typhoid model system, and has an altered LPS structure indicated by resistance to P22 phage and sensitivity to serum (22, 23, 32). LV386 is defective for the *rfc*-encoded polymerase and produces LPS with only a single O side chain (3). To confirm that the LPS produced by these strains was altered, LPS was resolved on SDS-Tricine gels (Fig. 6A), and immunoblot analysis was performed with the Sal4 monoclonal antibody specific for O-antigen factor 5 (Fig. 6B), or with polyclonal antibody directed against *S. typhimurium* O-antigen factors 1, 4, and 5 (Fig. 6C). LV386 produced LPS containing a single O-antigen

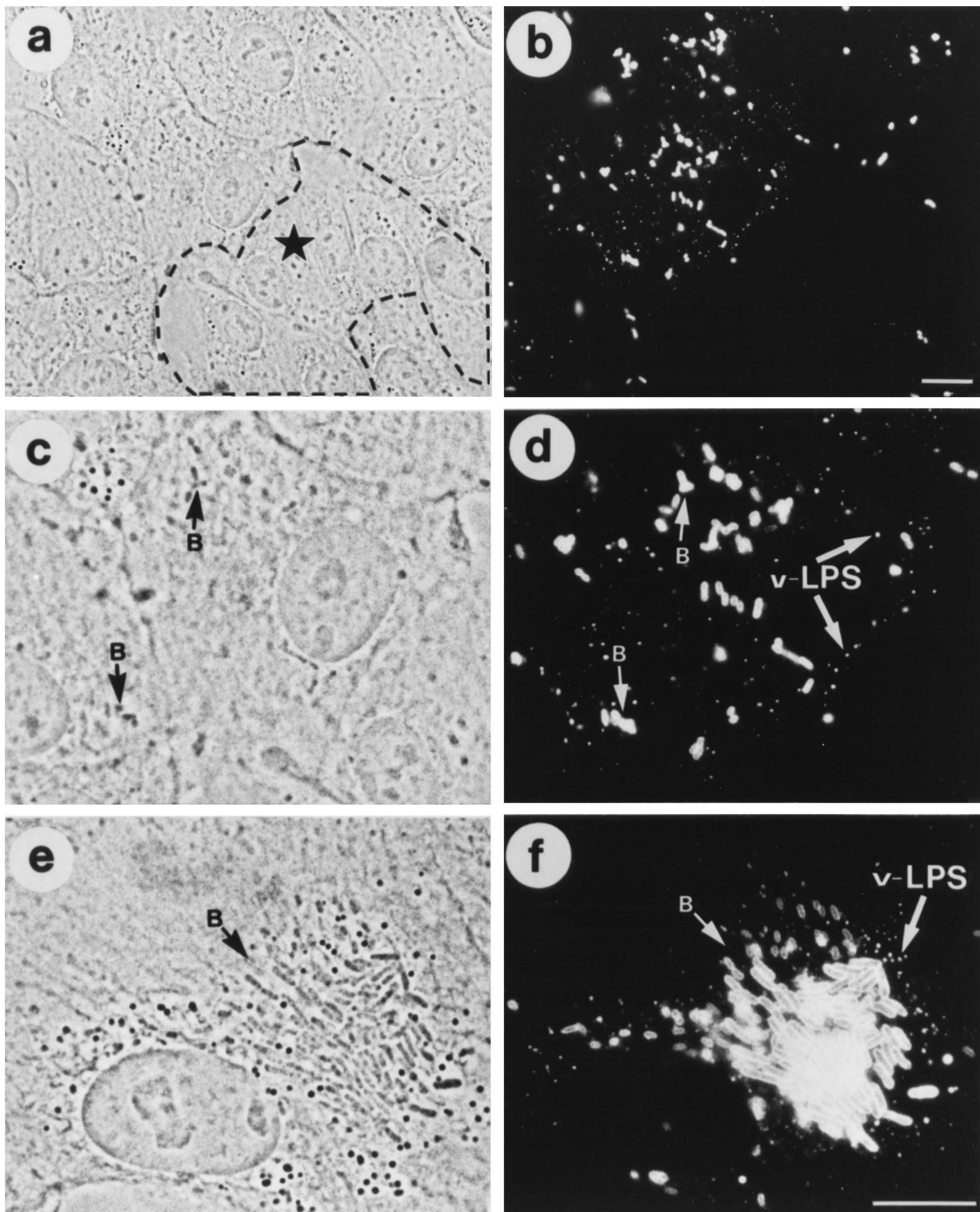

FIG. 2. Presence of vesicles containing LPS in the cytosol of MDCK epithelial cells infected with *S. typhimurium*. Epithelial cells were fixed and processed for immunofluorescence microscopy with the following primary antibody: polyclonal rabbit anti-*S. typhimurium* LPS (a to d) or the mouse monoclonal IgA anti-O-5 epitope of *S. typhimurium* LPS (e and f). (a and b) Low-magnification images of cells fixed at 2 h postinfection. Note that only infected cells show vesicles containing LPS, while neighboring uninfected cells (indicated by a star) show no LPS vesicles; (c and d) higher magnification of infected cells shown in panels a and b, showing the location of LPS-containing vesicles (v-LPS) and intracellular bacteria (B); (e and f) epithelial cells containing proliferating intracellular bacteria and fixed at 5 h postinfection, the time at which numerous vesicles containing LPS (v-LPS) are evident. Bars, 10 μm.

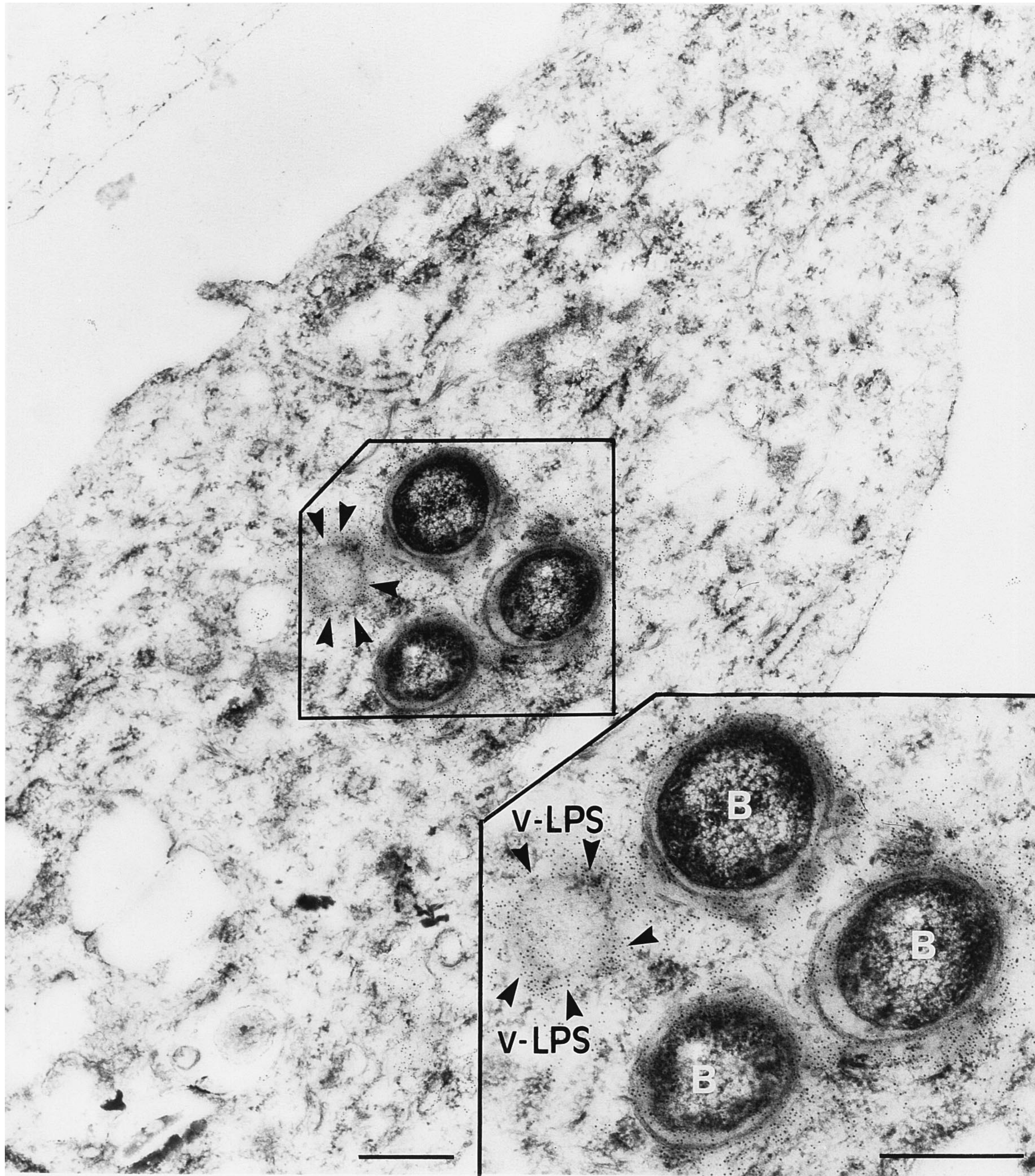

FIG. 3. Detection of LPS-containing vesicles by electron microscopy. HeLa epithelial cells were infected with *S. typhimurium* SL1344 and, at 6 h postinfection, processed for immunogold labelling. Polyclonal rabbit anti-*S. typhimurium* LPS and goat-anti-rabbit IgG conjugated with 5-nm gold particle were used as primary and secondary antibodies, respectively. The inset shows the presence of a vesicle containing LPS (marked v-LPS) beside intracellular bacteria contained in vacuoles (labelled B). Bars, 1 μm.

side chain. The bulk of 12-23 LPS is deficient for O antigen, but some high-molecular-weight O antigen is present (Fig. 6B and C). A quantitative enzyme-linked immunosorbent assay (33) revealed that both strains had approximately 1/10 of the O antigen as was contained in the same number of wild-type bacteria (data not shown). In contrast to the results obtained with the wild-type strain, 12-23 was unable to disseminate LPS throughout the host cytosol at any time postinfection (Fig. 7). Similar results were obtained for LV386 (data not shown). The reduced sensitivity of detection due to reduced O-antigen content in the mutant strain LPS must be considered a possible basis for this finding. However, the fluorescent intensity provided by antiserum binding to the 12-23 bacterial surface was indistinguishable from that in the wild-type strain SL1344 (compare Fig. 1, 2, and 7). Therefore, it is to be expected that at least some LPS-containing vacuoles would be detected in cells infected with these mutant strains if LPS were being disseminated.

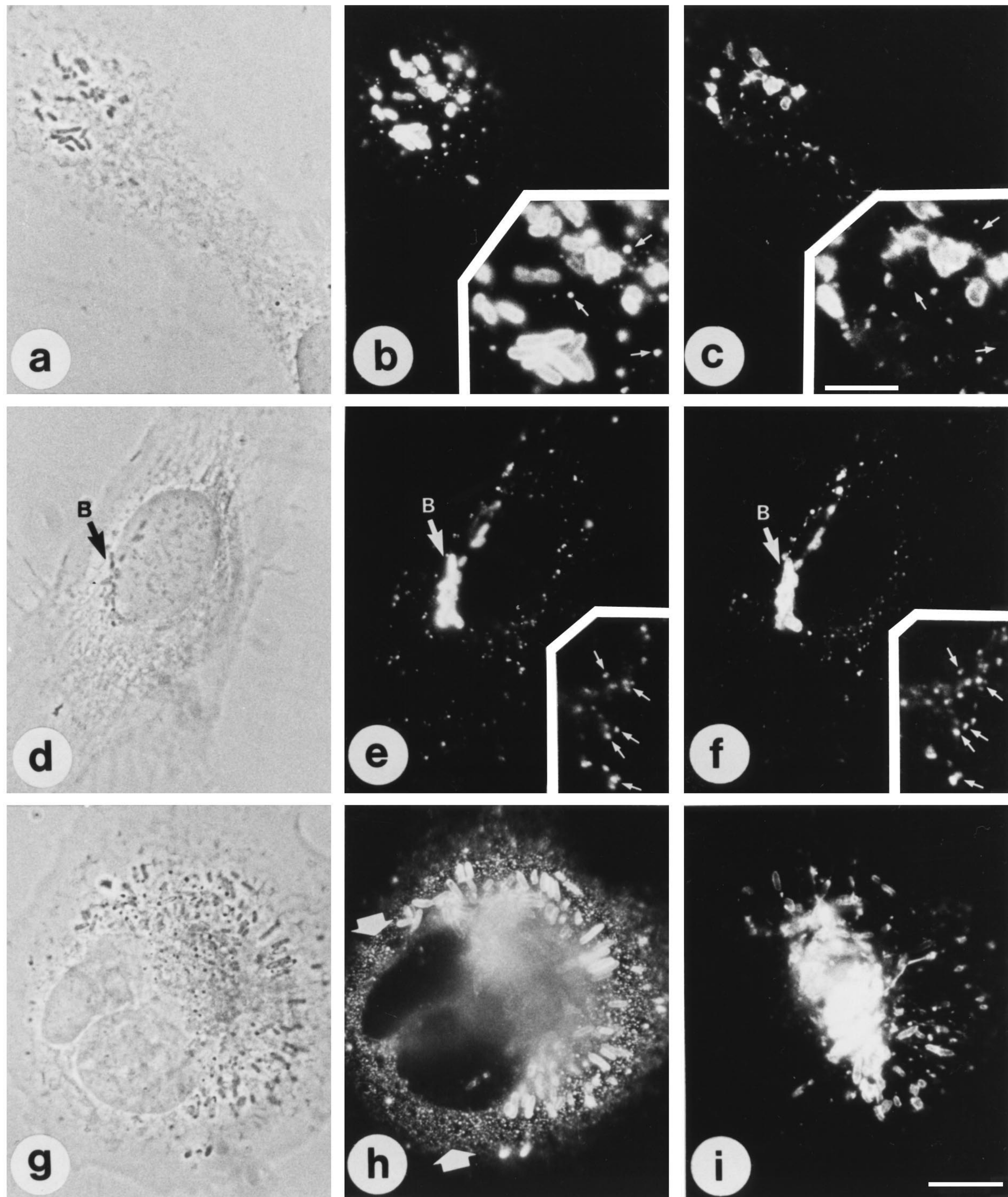

FIG. 4. Distribution of LPS-containing vesicles at different times postinfection. HeLa epithelial cells infected with *S. typhimurium* SL1344 were fixed at 30 min (a to c), 4 h (d to f), and 6 h (g to i) postinfection and processed for immunofluorescence analysis. Double labeling was performed with, as primary antibodies, rabbit polyclonal anti-*S. typhimurium* LPS antibody (b, e, and h) and mouse monoclonal H4B4 antibody, recognizing the human LGP h-lamp2 (c, f, and i). As secondary antibodies, FITC–goat anti-rabbit IgG and Texas red–goat anti-mouse IgG were used. Note the lack of colocalization of LPS and LGP at early times postinfection (small arrows, insets in panels b and c) and the colocalization of both markers in vesicles at later times (small arrows, insets in panels e and f). Numerous LPS-containing vesicles appear in the host cell cytosol when intracellular bacteria are proliferating (arrows in panel h). Bars, 10 μm.

Other described *S. typhimurium* mutants with altered LPS structures, such as the rough mutant 14028r (8), which has been reported to have a complete LPS core but no O side chain (8), were also assayed by immunofluorescence microscopy for intracellular release of LPS. Unfortunately, and in contrast to 12-23, the polyclonal antiserum anti-*S. typhimurium* LPS provided a very faint label on the bacterial surface, which was not sufficient to unequivocally establish an impairment on the LPS

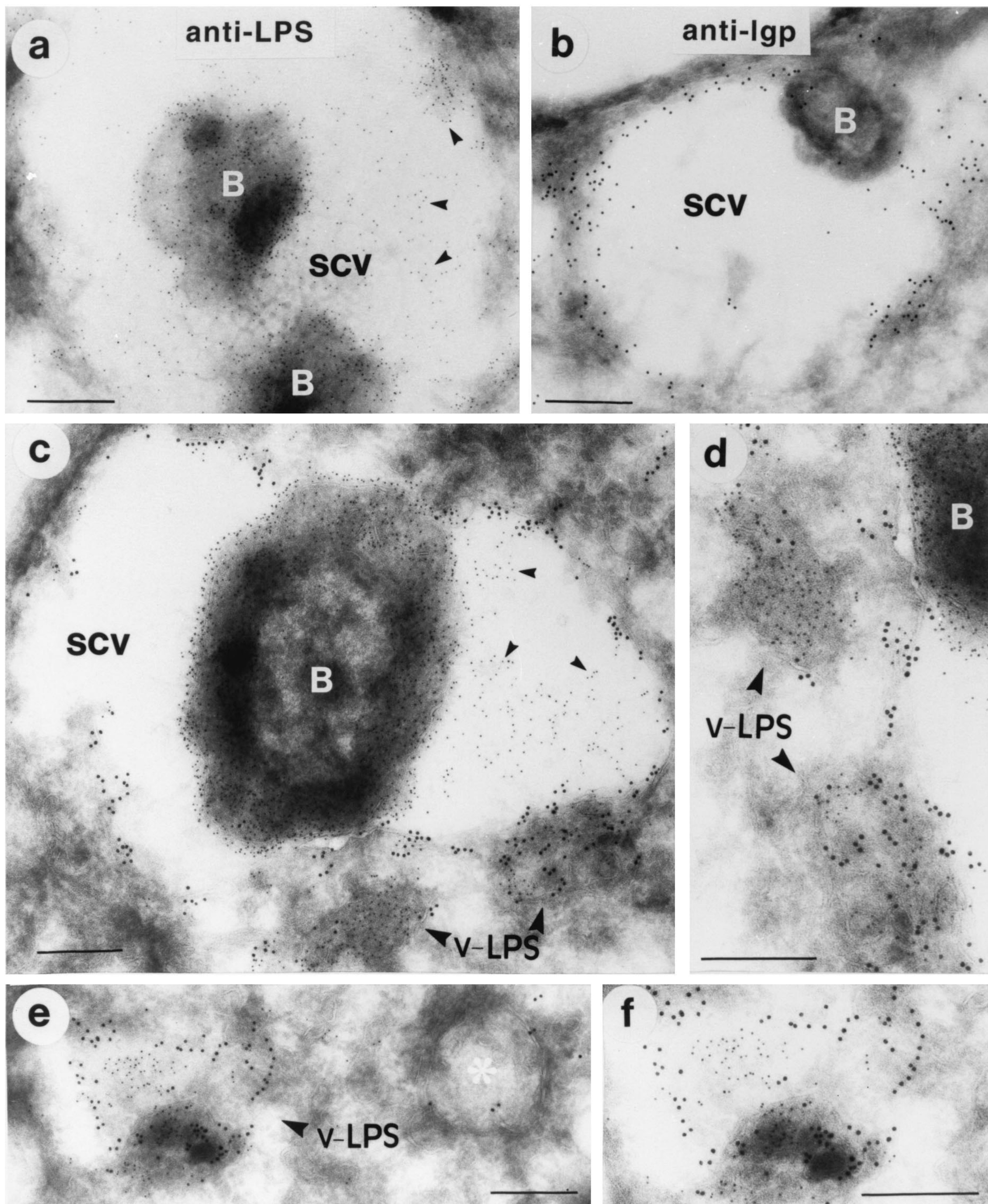

FIG. 5. Electron microscopy showing colocalization of LPS and LGP in SCV and vesicles present in the host cell cytosol (v-LPS). HeLa epithelial cells infected with *S. typhimurium* SL1344 were processed for immunogold labeling of thin cryosections at 4 h postinfection. (a) Single label of LPS with rabbit polyclonal anti-*S. typhimurium* LPS and goat anti-rabbit IgG conjugated to 5-nm gold particles. Arrows indicate LPS in the intravacuolar space and membrane of the SCV. (b) Single label of LGP with mouse monoclonal anti-LGP (anti-lgp in the figure) and goat anti-mouse IgG conjugated to 10-nm gold particles. LGP appears only in the membrane of the SCV. (c to f) Double labeling of LPS and LGP. (c) Typical distribution of LPS and lgp in an SCV. Small arrowheads indicate LPS in the intravacuolar space. Two v-LPS (large arrowheads) are observed close to the SCV. (d) Enlargement of the v-LPS shown in panel c containing both LPS and LGP. (e) Image of a vesicle containing LPS and LGP (v-LPS) present in the host cell cytosol. Note that there is another vesicle in the vicinity that is devoid of both markers (asterisk). (f) Enlargement of the v-LPS shown in panel e. B indicates intracellular bacteria. Bars, 0.5 μm.

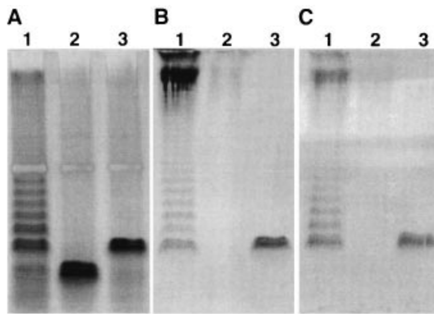

FIG. 6. LPS components recognized by the rabbit polyclonal anti-*S. typhimurium* LPS and mouse monoclonal antibody Sal4 anti-O-side chain. (A) Silver-stained LPS; (B) Western immunoblot with monoclonal antibody Sal4; (C) Western immunoblot with polyclonal antiserum. Lanes: 1, SL1344; 2, 12-23; 3, LV386 (*rfc*::*aph*). Note that both antibodies are able to recognize only the O-side chain units. 12-23 shows less high-molecular-weight O side chain units, which are detectable only with the antibodies, as well as an altered core with different electrophoretic mobility.

release process by this mutant. The very low fluorescent intensity obtained in strain 14028r agrees with our immunological data, which show a lack of recognition of the core region by this polyclonal antiserum (Fig. 6C). We also tested previously reported *S. typhimurium* mutants reduced for intracellular proliferation, such as 17-21 and 22-11 (prototrophic and purine-requiring intracellular replication mutants of SL1344) (22), for their capacity to induce intracellular release of LPS. These two mutants are susceptible to P22 phage infection (32) and therefore are not expected to have altered LPS structures. Both mutants released LPS intracellularly, suggesting that the release of LPS to the vacuolar system of the infected cell depends more on the LPS structure itself than upon extensive intracellular bacterial proliferation.

DISCUSSION

The results presented in this report show the capacity of an intracellular gram-negative bacterial pathogen, *S. typhimurium*, to export LPS from the intracellular compartment in which the bacteria reside. The appearance of LPS in host vesicles present in the cytosol resembles recent observations with another intracellular bacterial pathogen, *M. tuberculosis*, which is capable of releasing a surface component, the lipoarabinomannan, to vesicles which appeared scattered in the host cell cytosol (38). In our study, several lines of evidence demonstrate that *S. typhimurium* efficiently releases LPS by a process that occurs entirely within the intracellular environment. First, uninfected HeLa epithelial cells located in the vicinity of cells containing intracellular bacteria do not contain LPS-containing vesicles. Second, bacterial internalization is required for formation of vesicles containing LPS. Third, numerous LPS vesicles dispersed throughout the host cytosol do not appear in HeLa epithelial cells incubated with purified *S. typhimurium* LPS and treated to induce membrane ruffling and macropinocytosis, compared to *S. typhimurium*-infected cells. Finally, *S. typhimurium*-infected cells only rarely contain and never distribute intracellularly FITC-labeled *E. coli* LPS added simultaneously with invading *S. typhimurium.*

The export of LPS from compartments containing intracellular *S. typhimurium* seems to be a general process independent of the epithelial cell line used, as shown by the results obtained with HeLa and MDCK cells. Interestingly, the distribution and number of LPS-containing vesicles vary during the course of the infection. At 4 h postinfection, all intracellular *S. typhimurium* organisms are located in compartments containing eucaryotic lysosomal membrane glycoproteins (9, 11), and it is at this time when most of the LPS-containing vesicles also contain the eucaryotic marker, as shown by immunofluorescence and cryoelectron microscopy. Our study has also demonstrated that the number of vesicles containing LPS increases dramatically as the infection proceeds, reaching a maximum when bacteria are actively proliferating in the intracellular environment. Two alternative explanations may account for the enhanced release of LPS related to intracellular bacterial growth. First, intracellular bacteria may release LPS continuously from the time they enter the host cell. This assumption could indicate that the biosynthesis rate of LPS has to increase within the intracellular environment to maintain the integrity of the bacterial surface. An alternative explanation could be that part of the intracellular bacterial population dies, causing an additive effect for the LPS release process. This latter possibility seems unlikely, since there is no precedence for the

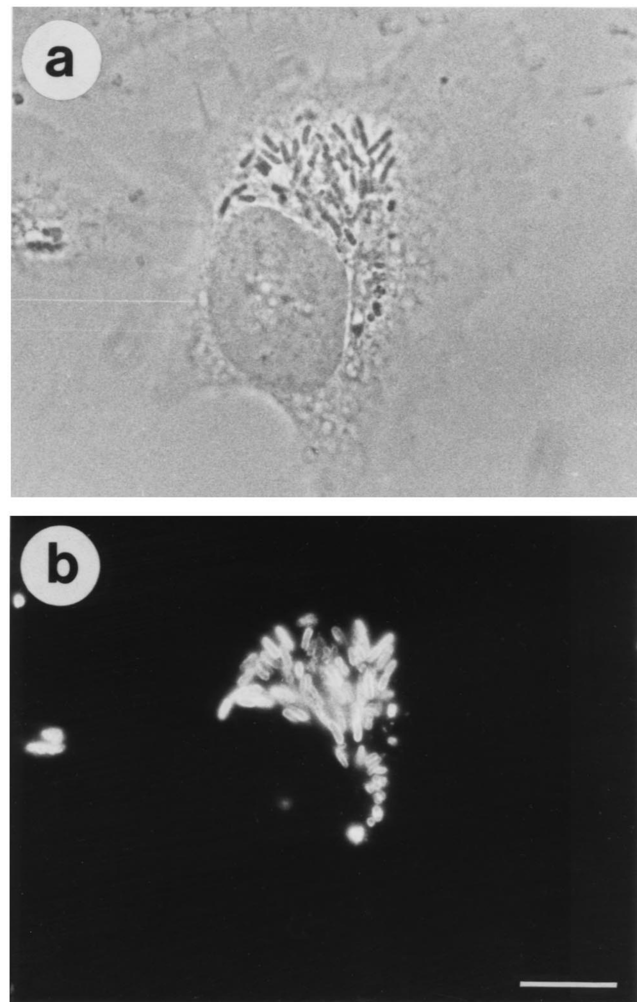

FIG. 7. Impaired intracellular release of LPS in HeLa epithelial cells infected with the avirulent *S. typhimurium* mutant 12-23. Cells were fixed at 6 h postinfection and processed for immunofluorescence microscopy with, as primary antibody, rabbit polyclonal anti-*S. typhimurium* LPS. (a) Phase-contrast image; (b) fluorescence image. Note the lack of vesicles containing LPS in the host cell cytosol. Identical results were obtained at other infection times (30 min and 2 and 4 h). Bar, 10 μm.

death of intracellular *S. typhimurium* organisms residing within epithelial cells.

How LPS is transported from the bacterial surface to the membrane of the SCV and vesicles located in the host cell cytosol is unknown. Signals in the intravacuolar environment may induce LPS release from the bacterial outer membrane. Released LPS can be further incorporated into the membrane of SCV, as suggested by the cryoelectron microscopy studies. Interactions between as yet undefined regions of the LPS molecule and eucaryotic components of the SCV may be required for the initiation of the LPS export process. This LPS can terminate in eucaryotic vesicles, originated in the SCV and formed as a result of vesicle budding processes inherent in every eucaryotic organelle (28). Transport of LPS from intracellular bacteria to the vacuolar membrane of SCV is analogous to the presence of LPS in the membrane of vacuolar compartments (symbiosomes) of amoebae containing X bacteria (19). There is also precedence for the transfer of bacterial LPS from one membrane to another during bacterial interaction with a host bacterial cell, as is the case for *E. coli* infection by *Bdellovibrio bacteriovorus* (33). Other investigations have also confirmed the incorporation of pathogen-derived molecules in the membrane of parasitophorous vacuoles containing parasites such as *Toxoplasma gondii* (1) or *Plasmodium falciparum* (7, 34) or the membrane of inclusion bodies containing *Chlamydia psittaci* (27). Nevertheless, our results with *S. typhimurium* in epithelial cells indicate an additional feature, as this pathogen is able to not only target LPS to the membrane of SCV but also induce the release of this molecule to vesicles distant from where intracellular bacteria are located.

Our findings from the analysis of LPS release by *S. typhimurium* LPS mutants suggest that proper LPS structure may be required for LPS release. The quantitative difference between the amount of O antigen present on the wild type compared to 12-23 or LV386 could explain the lack of detection of LPS-containing vesicles in the cytosol of HeLa cells infected with LPS mutant strains. However, alterations in the LPS and outer membrane structures (23) could be an alternative explanation supporting the impaired intracellular release of LPS by these mutants. No difference exists in signal between the wild-type strain and these two bacteria when they are labeled with the polyclonal antibodies for fluorescence microscopic analyses (Fig. 1, 2, and 7), and it would be expected that LPS-containing vesicles could be visualized in HeLa epithelial cells infected with these mutants.

Intracellular dissemination of LPS to host vesicles induced by *S. typhimurium* may play a significant role in activating specific types of cells, such as epithelial cells, which are not highly responsive to exogenous LPS. Epithelial cells do not contain membrane-bound CD14 (mCD14) or scavenger receptors, which are found primarily in myeloid-lineage cells and in macrophages respectively. These two type of membrane receptors play a key role in the interaction of LPS with eucaryotic cells (24). Thus, the scavenger receptor, located predominantly in liver macrophages, is involved in clearance of free LPS and promotes its uptake and further inactivation (13). On the other hand, mCD14 is involved in both LPS internalization and signaling of the host cell (12). Therefore, the release of LPS within epithelial cells could be considered to be a strategy used by *S. typhimurium*, and probably other intracellular bacterial pathogens, to locate this bacterial component intracellularly in a cell type which is apparently not designed to interact with exogenous circulating LPS. This strategy could also counterbalance the low internalization rate of LPS in epithelial cells, due to their relatively low endocytic activity compared to macrophages.

In this context, it is worth noting that the release of cytokine mediators has been found in epithelial cells infected with invasive pathogens such as *S. typhimurium* and *Listeria monocytogenes* (6). These and other results have supported an active signaling role for these type of cells (6, 17, 20). Interestingly, a recent study by Eckmann et al. (6) showed that interleukin-8 (IL-8) is secreted by epithelial cells infected with *S. typhimurium*, but this response is not reproduced by exogenous addition of LPS. This difference could be the basis of future work, designed to test whether part of the signaling process occurring in epithelial cells in response to bacterial infection could be linked to the release of bacterial components within the infected cell. The *S. typhimurium* mutant 12-23 used in the present study, which shows impaired capacity to release LPS intracellularly, could be assayed in these cytokine response studies. Finally, it will be also interesting to know whether, besides the O side chains present in the LPS-containing vesicles of the host cell, an additional part(s) of the LPS molecule is released by intracellular *S. typhimurium* and whether additional eucaryotic components present in intracellular locations, besides the β-tubulin and microtubule-associated proteins (5), have the capacity to interact specifically with LPS.

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