Trafficking of *Shigella* Lipopolysaccharide in Polarized Intestinal Epithelial Cells

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Abstract. Bacterial lipopolysaccharide (LPS) at the apical surface of polarized intestinal epithelial cells was previously shown to be transported from the apical to the basolateral pole of the epithelium (Beatty, W.L., and P.J. Sansonetti. 1997. *Infect. Immun.* 65:4395–4404). The present study was designed to elucidate the transcytotic pathway of LPS and to characterize the endocytic compartments involved in this process. Confocal and electron microscopic analyses revealed that LPS internalized at the apical surface became rapidly distributed within endosomal compartments accessible to basolaterally internalized transferrin. This compartment largely excluded fluid-phase markers added at either pole. Access to the basolateral side of the epithelium subsequent to trafficking to basolateral endosomes occurred via exocytosis into the paracellular space beneath the intercellular tight junctions. LPS appeared to exploit other endocytic routes with much of the internalized LPS recycled to the original apical membrane. In addition, analysis of LPS in association with markers of the endocytic network revealed that some LPS was sent to late endosomal and lysosomal compartments.

Key words: *Shigella* • lipopolysaccharide • transcytosis • trafficking • epithelial

The intestinal epithelium has a highly polarized structure with intercellular tight junctions defining two distinct domains, the apical surface facing the lumen and the opposing basolateral surface facing adjacent cells and the basement membrane (Louvard et al., 1992). A primary function of this, as well as any epithelial barrier, is to regulate the movement of macromolecules between the lumenal environment and the underlying tissue. The polarity of the epithelial monolayer associated with the presence of specialized apical and basolateral endocytic pathways controls selective exchanges of macromolecules across these cells. Components of the extracellular medium are internalized into membrane-bound vesicles and delivered to endosomes. Internalized membrane components, ligands, and fluid content within the endosomes are subsequently sorted for redistribution to specific destinations in the cell. Much of the endocytosed fluid and membrane components are recycled to the original plasma membrane surface (Knight et al., 1995). Some are transported to late endosomes en route to degradative lysosomes. In addition, molecules endocytosed by epithelial cells can be transcytosed to the opposite surface via a transcellular vesicular route (Mostov and Simister, 1985). Transcytosis is a key membrane trafficking process essential for the generation and maintenance of distinct cell surface domains and consequently a means for the exchange of macromolecules from one cell surface to the opposing surface (Schaerer et al., 1991).

The intestinal epithelium also plays a role as a protective barrier to lumenal threats. The intestine, and particularly the large bowel, is constantly exposed to bacteria and endotoxin due to natural colonization by numerous commensals. The proinflammatory endotoxin may penetrate to areas of tissue which could be poorly reached by the bacteria themselves. It was recently shown that lipopolysaccharide (LPS)[1] deposited at the apical surface of cultured polarized epithelial cells was recovered at the opposing basolateral side of the epithelium (Beatty and Sansonetti, 1997). This process occurred in concert with the proper establishment of intracellular tight junctions impli-

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1. *Abbreviations used in this paper:* CI-MPR, cation-independent mannose 6-phosphate receptor; FCS, flow cytometry standard; LAL, limulus amebocyte lysate assay; Lamp, lysosomal-associated membrane protein; LPS, lipopolysaccharide; MIIC, MHC class II.

cating a transcellular route in the apical to basolateral transit of LPS. LPS that trafficked across the epithelium retained the capacity to stimulate basolaterally positioned neutrophils to migrate into the paracellular space, indicating that transcytosis of this molecule in epithelial cells was not accompanied by its detoxification.

To elucidate the endocytotic routes of LPS, human colonic epithelial (T84) cells were used. Polarized T84 cells exhibit remarkable resemblance to native colonic crypt cells, forming tight junctions and a well-defined brush border at their apical membrane, and allowing for vectorial electrolyte transport (Madara and Dharmsathaphorn, 1985). These cells served as the model for quantitation and characterization of LPS internalization, recycling, and apical to basolateral transcytosis. The intracellular distribution of LPS deposited at the apical surface was analyzed in relation to markers that define the major endocytic pathways. The intracellular routes exploited by LPS may have important implications on the dynamic role of the intestinal epithelium not only as physical barrier, but an intermediary between luminal microbial threats and the underlying protective innate and specific immune systems.

Materials and Methods

Cell Culture

T84 colonic intestinal epithelial cells were maintained in culture medium (1:1 of Dulbecco-Vogt modified Eagle's medium and Ham's F12 (GIBCO BRL) supplemented with 10% newborn calf serum, 10 mM Hepes buffer, 40 μg/ml penicillin, and 90 μg/ml streptomycin. Polarized epithelial cell monolayers were prepared by seeding 5×10^5 cells on Transwell-Clear polyester membranes (3-μm pore size, 6.5-mm-diam Costar inserts; Costar Corp.). Integrity of tight junctions was monitored by transepithelial resistance to passive ion flow using a dual voltage ohmmeter (Millicell-ERS; Millipore Corp.). Day 5 monolayers with transepithelial resistance of 1,500–2,000 Ω/cm^2 were used in this study.

Reagents

LPS was extracted and purified from invasive *S. flexneri* M90T according to Westphal (Westphal and Jann, 1965). Lyophilized LPS was resuspended in tissue culture medium to the indicated concentration and sonicated lightly before use. Iron-saturated holo-transferrin, fatty acid-free BSA fraction V, FITC, and TRITC were obtained from Sigma Chemical Co. Transferrin-FITC, BSA-FITC, and BSA-TRITC were prepared essentially as described by Goding (1976). Anti–CI-MPR (cation-independent mannose 6-phosphate receptor) antibody was a gift of Dr. Bernard Hoflack (IBL, Lille, France). Anti–Lamp 2 (lysosomal-associated membrane protein) (CD3) antibody was a gift of Dr. Minoru Fukuda (La Jolla Cancer Foundation, La Jolla, CA). Anti-canine rab7 antibody was raised against a synthetic peptide corresponding to the COOH terminus of the protein (residues 172–204) and covalently coupled to keyhole limpet hemocyanin. Antibodies to *S. flexneri*-5a LPS were a kind gift of Dr. Armelle Phalipon (Institut Pasteur, Paris, France).

Kinetics of LPS Transcytosis and Recycling

To quantitate LPS recycling at the apical surface in addition to transcytosis, 2 μg/ml of LPS was added to the apical pole and cells were incubated for 2 h at 37°C. The filters were then placed on ice and the apical and basal surfaces were washed extensively with cold culture medium to remove extracellular LPS. LPS was quantitated by ELISA (sensitive to 1 ng/ml) and confirmed by modification of the chromogenic limulus amebocyte lysate assay (LAL; sensitive to 10 pg/ml; Biowhittaker Inc.). The final wash revealed no LPS by ELISA (negligible by LAL assay). Prewarmed LPS-free medium was then added to both reservoirs and monolayers were incubated at 37°C. The integrity of intercellular tight junctions was maintained after LPS treatment as confirmed by transepithelial resistance and absence of paracellular passage of ^{3}H-mannitol. At the times indicated, the cells were rapidly cooled on ice, apical and basal supernatants were collected. Monolayers were subject to three cycles of freeze/thaw followed by light sonication. Corresponding LPS levels in these fractions were quantitated as described above.

Ultrastructural Analysis of LPS and LPS/Transferrin Transcytosis

For initial description of LPS transcytosis, purified LPS was conjugated to 10 nm colloidal gold particles as previously described (Kriegsman et al., 1993). Gold complexes were stabilized with 2% polyethylene-glycol (average mol wt = 6,000–8,000). LPS-gold was added to the apical side of polarized T84 cells for 2 to 6 h. Monolayers were then fixed with 1.6% glutaraldehyde in 0.1 M phosphate buffer for 1 h, post-fixed with 2% osmium tetroxide for 1 h, and embedded in Epon-Araldite. Thin sections were obtained, conventionally stained and observed with a Philips CM12 electron microscope.

Subsequent analysis of LPS transcytosis was performed using differential immunogold labeling of LPS and of the transferrin receptor. LPS was added to the apical side of polarized T84 cells at a concentration of 25 μg/ml for 2 h. Cells attached on filters were fixed with 3% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 1 h at room temperature. Free aldehydes were quenched with PBS and 50 mM NH_4Cl for 15 min. Cells were dehydrated with an increasing ethanol series at 4°C and infiltrated in LR White. Blocks were polymerized at 50°C for 24 h (Newman and Hobot, 1993). Thin sections were incubated in drops of the following solutions: 10 min in PBS, 1% BSA, 1% NGS (normal goat serum), 0.2% Tween 20; then 10 min in a solution containing a rabbit immune serum against *S. flexneri* serotype 5a antigen at a 1/100 dilution and a mouse monoclonal antibody (H 68.4) directed against the human transferrin receptor (ZYMED Laboratories Inc.) at a dilution of 1/50 in PBS, BSA 0.1%; then 5 min in a solution containing an anti–rabbit IgG, gold-conjugated (10 nm beads) serum (British Biocell International) and an anti–mouse IgG (H+L) gold-conjugated (5-nm beads) serum (British Biocell International), both diluted 1/20 in PBS, 0.01% gelatin (Sigma Chemical Co.). Two 5-min washes were then performed in PBS and one 5-min wash was performed in PBS, 1% glutaraldehyde. Thin sections were then washed thoroughly with distilled water and counterstained with 1% uranyl-acetate (5 min). Observations were made at 60 kV with a Philips CM12 electron microscope.

Analysis of LPS by Confocal Microscopy

For uptake experiments, polarized cells were briefly rinsed with prewarmed culture medium. LPS (2 μg/ml) or BSA-TRITC (10 mg/ml) was added to the apical side of the epithelium for 20 min at 37°C. For analysis of longer internalization times, cells were further incubated in LPS- or BSA-free medium for an additional 40 min or 1 h and 40 min, corresponding to 1 and 2 h total internalization time, respectively. For basolateral uptake, transferrin-FITC (30 μg/ml) or BSA-FITC (10 mg/ml) was added simultaneously to the opposing basolateral pole of the polarized epithelium for the final 10 min of internalization.

Apical and basolateral sides of cell monolayers were carefully washed with PBS containing 0.2% BSA at 4°C. After a final wash with PBS, cells were fixed for 30 min at room temperature in 3.7% paraformaldehyde in PBS, pH 7.4. Filters were then rapidly washed in PBS, incubated in 50 mM NH_4Cl in PBS for 15 min and either directly mounted on coverslips in PBS containing 50% glycerol or further processed for immunofluorescence. For this purpose, cells were permeabilized with two rapid washes of PBS and 0.1% saponin and incubated for 30 min with primary antibodies in PBS and 10% horse serum and 0.1% saponin. Filters were extensively washed with PBS and 0.1% saponin, incubated for 30 min with secondary antibodies, washed, mounted on coverslips and viewed under a Leica TCS 4DA confocal microscope. Series of two plane sections of 1 μm thickness were monitored. For double-staining experiments, identical optical sections are presented. Superimposed images were treated with a pseudocolor scale. Colocalized structures are seen in a yellow color.

Multifluorescence Image Cytometry of Confocal Micrographs

A new multifluorescence image cytometry approach (Demandolx and Davoust, 1997) was used to estimate the proportion of vesicles containing internalized LPS and marked with antibodies raised against different intracellular markers. To identify all labeled vesicles, the high spatial fre-

quency noise was removed from digital confocal images using a Gaussian convolution filter (SD of 2 pixels) followed by a top-hat based subtraction (radius of 9 pixels) and a grey level segmentation (20% of maximum intensity). To provide a statistical analysis of attributes of identified objects (size, fluorescence densities in channels FL1 and FL2), image cytometry file were created, stored in flow cytometry standard (FCS) and analyzed using CellQuest software (Becton Dickinson). For all objects, we determined the percentage of LPS-containing vesicles that were positive for the other marker on 2D-scatterplots representing the fluorescence FL1 vs FL2.

Results

Apical LPS Internalization, Transcytosis, and Potential Recycling

Previous studies have shown that when 0.8 μg/ml of LPS, a concentration equivalent to the amount of LPS spontaneously released by 10^8 *S. flexneri*, is present at the apical surface of polarized T84 cells, LPS retaining endotoxic activity is recovered at the basolateral side of the epithelium (Beatty and Sansonetti, 1997). This process occurred in monolayers with properly established intercellular tight junctions, indicating that LPS was directed basolaterally via a transcellular route. To analyze the kinetics and quantify transcellular movements of LPS, LPS at a concentration of 2 μg/ml was added to the apical pole of polarized T84 cells for 2 h at 37°C. Monolayers were then washed extensively at 4°C to remove extracellular LPS and then reincubated at 37°C in LPS-free medium for the indicated times. As shown in Fig. 1, 119 ± 17.8 ng of LPS was associated with the epithelial cell monolayer after the 2-h loading period. With subsequent culture in LPS-free medium, LPS was transcytosed to the basolateral pole, however, the bulk of LPS was released at the apical surface of the polarized monolayer. After 1 h, 17.7 ± 3.6 ng of LPS was released basolaterally corresponding to 15% of the total LPS initially loaded (Fig. 1). This level is sufficient to stimulate clinically relevant biological activities. 45.4 ± 7.5 ng of LPS, equivalent to 38% of the initial cell-associated LPS, was detected in the apical reservoir. Apical LPS may correspond to aggregated LPS at the apical surface which was subsequently released without internalization. However, microscopic analyses revealed that under these conditions cell-associated LPS was primarily intracellular with a minute amount remaining membrane-associated. These results therefore suggest that after internalization, the LPS was recycled back to the apical pole, as well as transcytosed basolaterally. In addition, the total amount of detectable LPS preloaded into the epithelial cells declined by 38% after 30 min of subsequent culture in LPS-free medium. This loss of LPS, as detected by ELISA and the LAL assay, may reflect LPS transport and possible degradation in the lysosomal compartments.

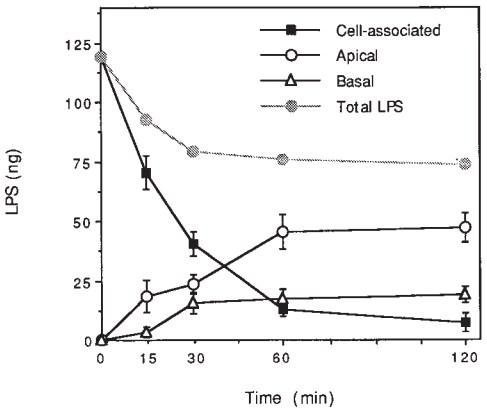

Figure 1. Kinetics of LPS released at the apical and basolateral surfaces of polarized epithelial cells. T84 cell monolayers were preloaded for 2 h with 2 μg/ml of LPS at the apical pole. After subsequent washing to remove extracellular LPS, the fate of the cell-associated LPS was analyzed at the times indicated. The results are expressed as the mean ± SD of three individual monolayers.

Morphological Analysis of LPS Uptake and Transcytosis

For conceptual purposes, the route of LPS movement through the polarized epithelium was analyzed by transmission electron microscopy. LPS-coated colloidal gold particles were placed on the apical side of polarized T84 cells. Analysis of LPS association with the epithelium revealed LPS within coated pits and coated vesicles at the apical membrane suggestive of uptake by receptor-mediated endocytosis (Fig. 2). Intracellularly, LPS concentrated within apical multimembranous bodies immediately below the brush border (Fig. 3 a). LPS was rarely observed in the perinuclear region and labeled particles were not present in the basolateral region below the nucleus. Because the intactness of tight junctions was maintained as monitored by electrical resistance and restriction of mannitol passage, the lack of any labeling in the basolateral region implicated the paracellular space below the tight junctions as a possible target for LPS transcytosis. Supporting this premise, labeled vesicular structures in close proximity of the paracellular space were observed with apparent exocytosis of LPS-coated particles into the paracellular space (Fig. 3, e and f).

LPS Accesses a Basolateral Compartment

To analyze the pathway traversed by apically internalized LPS and its mode of access to the basolateral medium, confocal microscopy and transmission electron microscopy were employed. As observed in confocal microscopy, the height of the polarized T84 cells was ~30 μm. Three representative planes in each focal series were selected to analyze the distribution of LPS over the entire cell height. The three focal planes are illustrated in Fig. 4 A, scanning the cells through the apical, medial, and basolateral region of the polarized T84 cells. The basolateral surface of polarized T84 cells extends up to the paracellular tight junctions, ~0.5 μm from the apical plasma membrane. Because ultrastructural analysis revealed that internalized LPS was confined to vesicles in the apical region, the accessibility of LPS to basolateral endosomes was analyzed. LPS was internalized from the apical side for 20 min. Transferrin receptors are located on the basolateral membrane of differentiated cells (Fuller and Simons, 1986), therefore transferrin, a protein which is endocytosed and recycled at the basolateral surface, was subsequently added to the opposing basolateral pole for the last 10 min

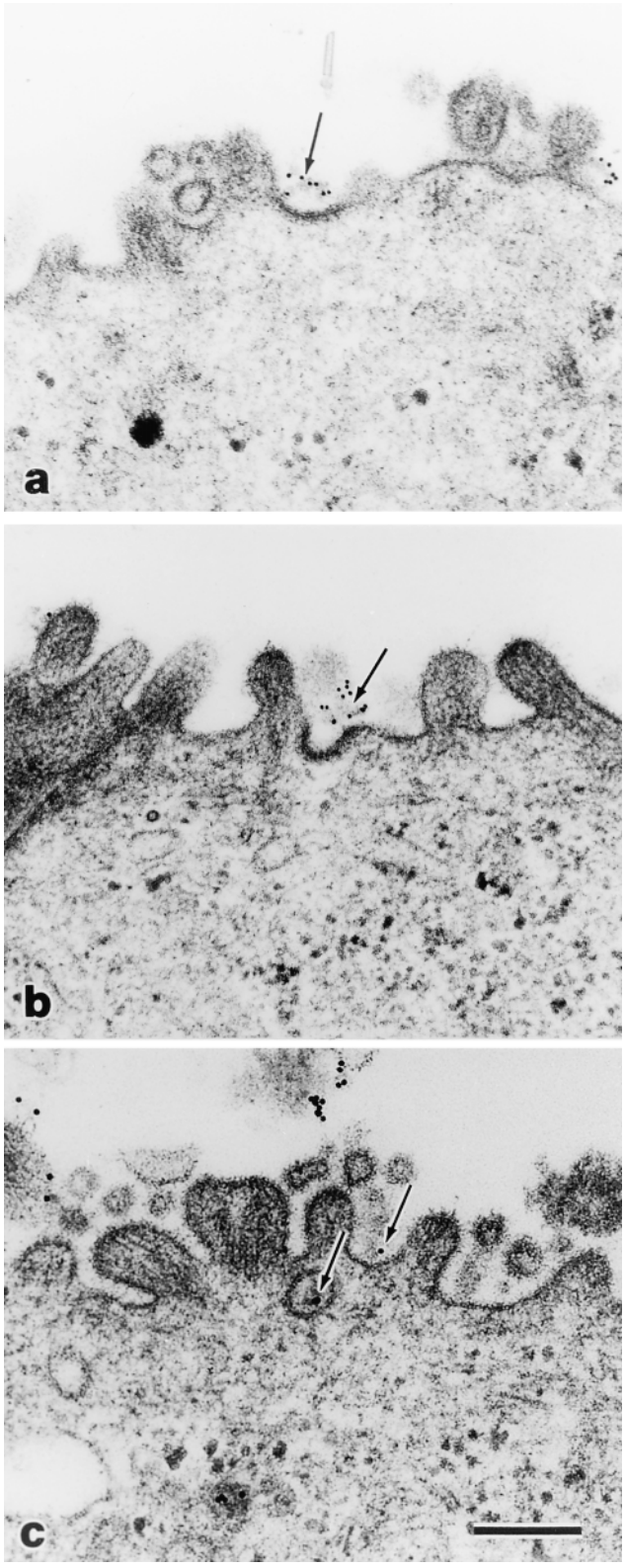

Figure 2. Analysis of LPS interaction with the apical surface of polarized T84 cells by electron microscopy. After internalization of LPS-coated colloidal gold at the apical plasma membrane, these particles (arrows) were found in association with coated pits (a–c). In addition, LPS-coated particles could be localized to coated vesicles at the apical surface (c). Bar = 200 nm.

of internalization serving as a marker for basolateral early endosomal compartments. As shown in Fig. 4 A, transferrin was present in all focal planes. Consistent with ultrastructural analysis, LPS was evident only in the apical region. Superimposed images revealed striking colocalization of LPS to transferrin positive vesicles. Analysis of confocal micrographs by image cytometry indicated that >80% of LPS-containing vesicles also contain transferrin (Table I). These results suggest that LPS trafficks to basolateral endosomes and may access the basolateral side of the epithelium via exocytosis into the paracellular space beneath the tight junctions.

Transmission electron microscopy confirms confocal images, showing vesicles with internal multimembranous structures containing both apically internalized LPS (gold size 10 nm) and the transferrin receptor (gold size 5 nm), indicating that LPS has undergone a transcytotic process from the apical to the basolateral domain of T84 cells (Fig. 3, b–d). These vesicles are essentially located at the upper part of T84 cells, close to the lateral membrane.

The LPS Transcytotic Compartment Is Not Readily Accessible to Fluid-Phase Markers

To further define the basolateral transferrin compartment that was accessible to LPS, the fluid-phase marker BSA was analyzed. BSA was added to the apical surface of cells for 20 min and transferrin was subsequently added basolaterally for the final 10 min of internalization. As observed with LPS, apically internalized BSA was present only in the apical plane of T84 cells (Fig. 5). In contrast, BSA failed to colocalize with basolaterally internalized transferrin. In addition, when LPS was added apically and the fluid-phase marker added to the opposing basal pole, a meager level of colocalization was observed (Fig. 5). Thus within the first 20 min of internalization, LPS was sorted to an endosomal compartment accessible to basolaterally internalized transferrin and from which fluid-phase material was essentially excluded.

LPS Is Transported to Late Endosomes and Lysosomes

To determine its subsequent fate, LPS was internalized apically for 20 min followed by a 40-min chase with LPS-free medium. As shown in Fig. 4 B, LPS remained in the apical plane with slight levels moving to the medial perinuclear region (not shown). At this internalization time, most of LPS no longer colocalized with basolaterally loaded transferrin. The distribution of LPS distinct from transferrin-containing compartments was even more evident after 2 h of internalization (20 min LPS internalization followed by 1 h 40 min chase) and only 10% of LPS-positive structures were shown to contain transferrin (Table I). These results could indicate that LPS is exocytosed at the lateral surface remaining trapped within the paracellular space. Despite the likelihood of this possibility based on the kinetic analyses described above, and electron microscopy revealing exocytosis of LPS into the paracellular region (Fig. 3), the punctate pattern of LPS labeling (Fig. 4 B) suggests that extended internalization of LPS results in the sorting of this molecule to vesicles independent of transferrin labeling.

To further characterize compartments to which LPS is

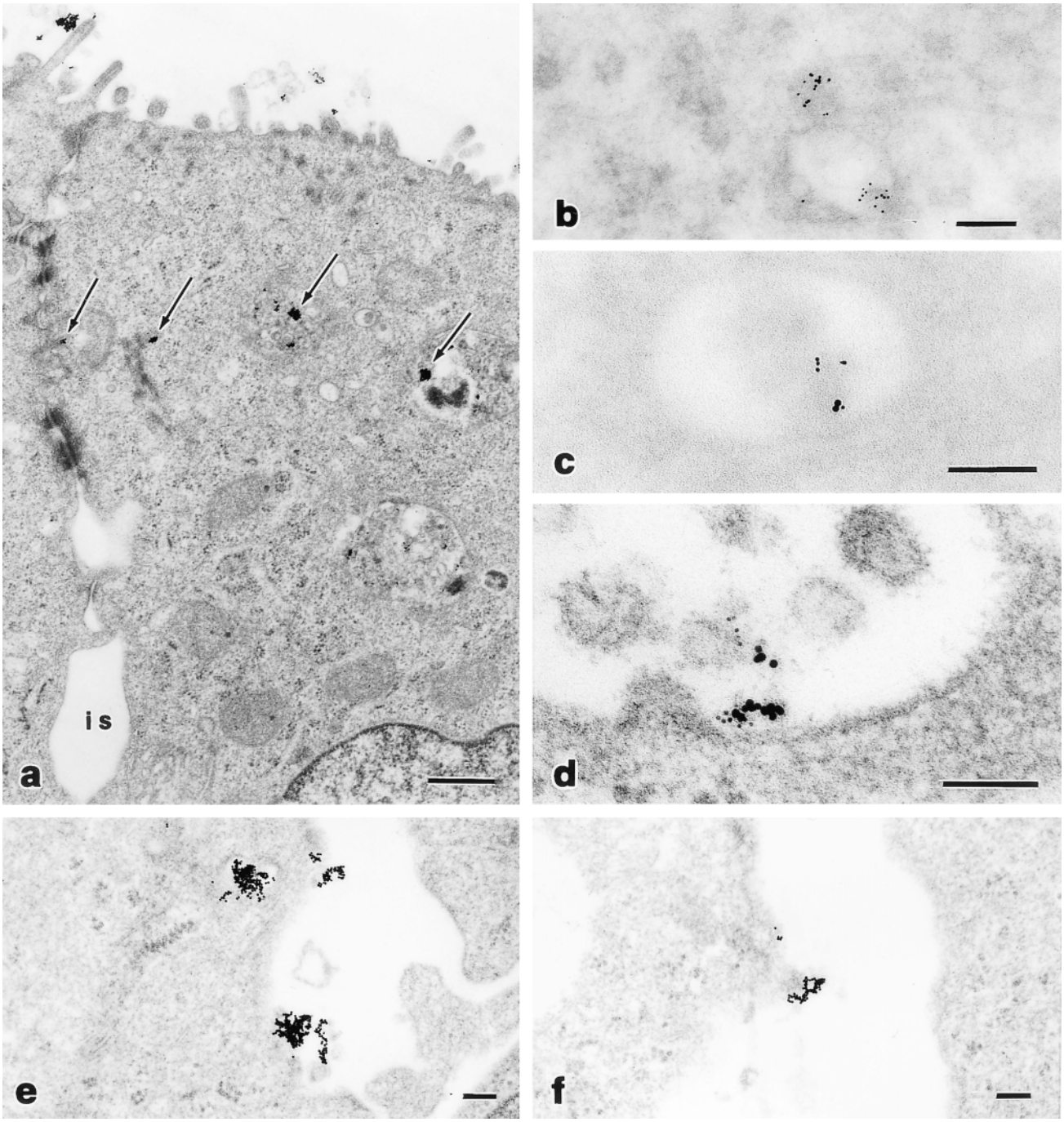

Figure 3. Analysis of transcytosis of LPS by transmission electron microscopy. (a, e, and f) Cross-section of a polarized T84 cell monolayer, after internalization of LPS-coated gold particles at the apical pole. Panel a shows apical villi, tight junctions, inter(para)cellular space (i.s.), and basally positioned nucleus. LPS-coated particles primarily localize to vesicles in the apical region of the polarized cell (arrows), some of them in close proximity of the lateral region opposing the paracellular space. e and f show the intercellular region below the tight junctions, with LPS appearing within basolateral endosomes adjacent to the lateral membrane and proceeding to exocytosis into the paracellular space. (b–d) Double immunogold labeling of LPS (10 nm gold particles) and the transferrin receptor (5 nm gold particles). Panel b shows two adjacent vesicles, one containing the transferrin receptor alone (bottom) and one (top) containing both LPS and the transferrin receptor. Panels c and d show multimembranous vesicles containing both LPS and the transferrin receptor. Bars: (a) 500 nm; (b–f) 100 nm.

transported, markers of the endocytic network were incorporated into this study. When internalized apically for 20 min, LPS failed to colocalize with the late endosomal marker CI-MPR (Fig. 6, Table I). However, a longer internalization of 1 h (20 min LPS internalization followed by 40-min chase) revealed dramatic colocalization of LPS with the CI-MPR, a well accepted marker for late endosomal compartments, in the apical region of these cells. Ex-

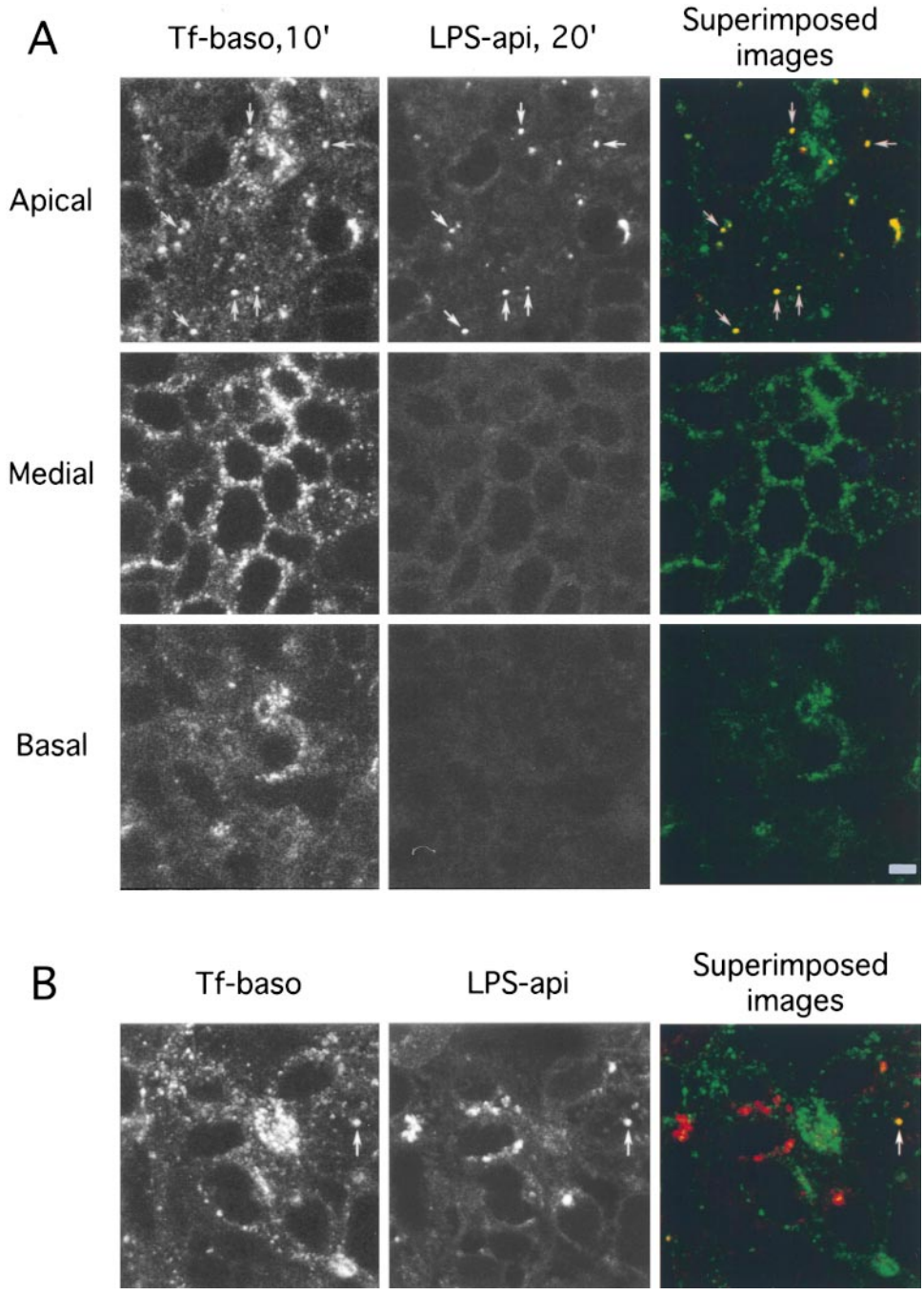

Figure 4. Apically internalized LPS localized to a basolateral transferrin-containing compartment. (A) LPS was internalized for 20 min from the apical domain of polarized T84 cells (column 2). Transferrin-FITC (Tf) was added to the basolateral pole for the last 10 min of internalization (column 1). Consecutive horizontal planes in a confocal series are shown representing the apical (2 μm from plasma membrane), medial (10 μm below the apical scan), and basal (2 μm above the filter) region of the epithelial monolayer. The corresponding images of each plane were superimposed in a double-color image in which LPS appears in red and the pattern of transferrin labeling is in green (column 3). Arrows indicate LPS and transferrin colocalization in the apical region of the cells, these structures being yellow in color when the images are superimposed (column 3). (B) LPS was added to the apical pole of polarized T84 cells for 20 min followed by incubation in LPS-free medium for an additional 40 min (column 2). Transferrin-FITC was added at the basolateral surface for the final 10 min of internalization (column 1). LPS remained in the apical region of the polarized cells, thus only the apical plane is shown. Superimposed images (column 3) revealed that colocalization between LPS and transferrin (as indicated by the arrow) declined with extended time of LPS internalization. Bar, 5 μm.

tension of LPS internalization to 2 h (20 min with 1-h 40-min chase) resulted in very low colocalization between these molecules. In addition, analysis of colocalization with rab 7 revealed a similar pattern of trafficking to late endosomes (not shown).

Access of LPS to lysosomes was analyzed by colocalization studies with the lysosomal glycoprotein Lamp 2. Using the same time frame of internalization, LPS access to Lamp-containing vesicles was evident after 1 h, with striking localization of LPS to lysosomes after 2 h of LPS internalization (Fig. 6, Table I). These results show that LPS is transiently associated with late endosomes and accumulates in lysosomes. It also indicates that the loss of detectable cell-associated LPS after internalization as shown in Fig. 1, is likely a consequence of LPS processing in lysosomal compartments.

Discussion

The polarized intestinal epithelium possesses two functionally distinct plasma membrane domains that intercede opposing forces. The apical surface counters microbial threats and the presence of numerous commensals, being bathed in bacterial products such as LPS which has the capacity to stimulate proinflammatory immune responses. The basolateral surface interfaces the underlying immune

Table I. Kinetic Analysis of Double-labeled LPS-containing Vesicles by Image Cytometry

Chase Time*	Percentage of LPS-positive vesicles double labeled with the marker‡		
	Tf	CI-MPR	Lamp 2
min			
0	82 ± 9	1	8
40	27 ± 4	84 ± 4	24
100	10 ± 2	6 ± 1	78 ± 5

*LPS was internalized for 20 min from the apical side of polarized T84 cells, chased for the indicated time. Cells were counter labeled with basolaterally internalized transferrin-FITC or CI-MPR or Lamp 2 as described in Materials and Methods.
‡Images representing a field of 100 × 100 μm were analyzed by subcellular cytometry as described in Materials and Methods. After background subtraction and top-hat–based segmentation of each channel, a mean of 51 ± 22 LPS-positive structures was analyzed per field. Values represent the result of a single image analysis or the mean of duplicates ± SD.

system serving as a substrate for resident and emigrating cells that provide mucosal protection. Signaling between the apical and basolateral domains may occur in response to microbial-induced upregulation of cytokines or ligands of the epithelial cells allowing for emigration and enhanced interaction of immune cells at the basal pole. In addition, interaction between the two domains may occur via vectorial delivery of molecules across the polarized monolayer by transcytosis, a multistep vesicular transport pathway (Schaerer et al., 1991). Transcytosis reflects a dynamic role of the intestinal epithelium in attuning these opposing forces.

After internalization at the apical or basolateral domain, much of the endocytosed fluid, macromolecules, and membrane components are recycled to the original plasma membrane surface (Bomsel et al., 1989; Godefroy et al., 1990; Knight et al., 1995). Endocytosed material may be sorted in early endosomes to late endosomes and lysosomes, or to transcytotic vesicles for delivery to the opposing surface. How molecules move from one early endocytic compartment to the opposite cell surface remains unclear. Numerous studies suggest that the apical and basolateral endocytic pathways interconnect at an apically localized transcytotic compartment (Hughson and Hopkins, 1990; Apodaca et al., 1994; Barroso and Sztul, 1994; Knight et al., 1995; Odorizzi et al., 1996). Basolaterally internalized ligands accumulate in this apical compartment, accessible to apically internalized membrane markers (Hughson and Hopkins, 1990; Apodaca et al., 1994; Knight et al., 1995; Odorizzi et al., 1996). Although this compartment can be accessed by basolaterally endocytosed transferrin that recycles back to the basolateral surface (Hughson and Hopkins, 1990), it is thought to be distinct from the basolateral transferrin recycling pathway, as well as basolateral early endosomes and the lysosomal network (Barroso and Sztul, 1994).

LPS released from *S. flexneri* present on the apical side of polarized epithelial cells was detected in the basolateral milieu under conditions in which the integrity of intercellular tight junctions was maintained (Beatty and Sansonetti, 1997). The uptake and intracellular endocytic processing of LPS was analyzed to elucidate the transcytotic pathway. LPS was internalized at the apical surface, clustered into coated pits and subsequently coated vesicles, suggestive of uptake via receptor-mediated endocytosis. Although a receptor for LPS in epithelial and endothelial cells has not been identified, binding studies have implicated the existence of such a receptor (Aracil et al., 1985;

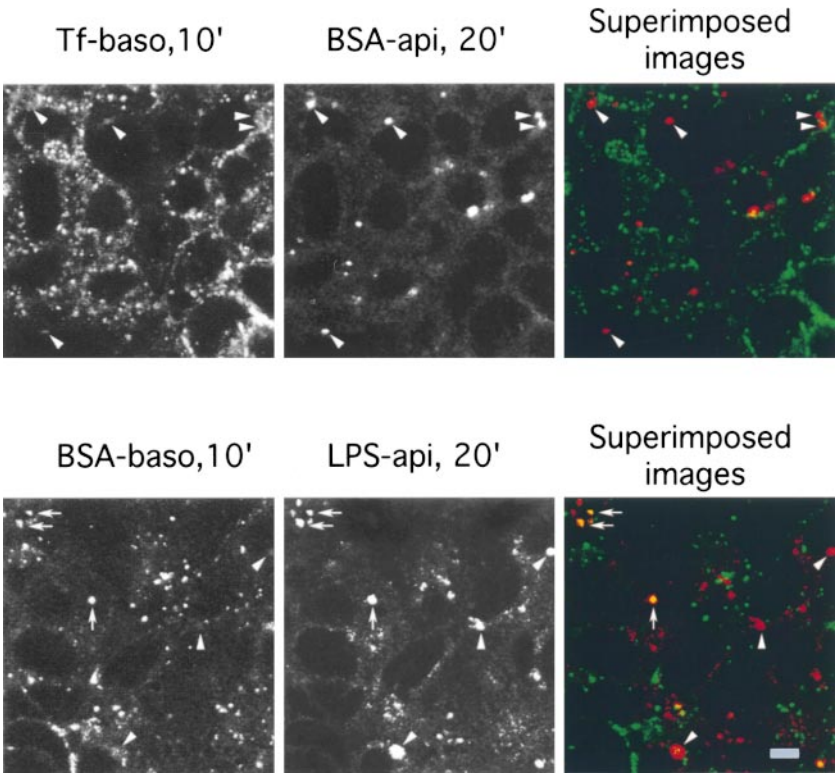

Figure 5. Fluid-phase markers followed an endocytic pathway distinct from LPS. (Top) BSA-rhodamine was internalized for 20 min from the apical domain of polarized T84 cells and transferrin-FITC (Tf) was added to the basolateral side of the epithelium for the last 10 min of internalization. Apical BSA did not colocalize with basolateral transferrin (arrowheads). (Bottom) LPS was added apically for 20 min and BSA-FITC was added basolaterally for the final 10 min of internalization. The majority of apical LPS did not colocalize with basolateral BSA (arrowheads). Arrows show limited colocalization between LPS and BSA. Analysis of cells resulted in localization of fluorescent molecules within the apical region of polarized cells, therefore only the scan corresponding to a plane 2 μm from the apical surface is shown. Column 3 illustrates the superimposed images. Bar, 5 μm.

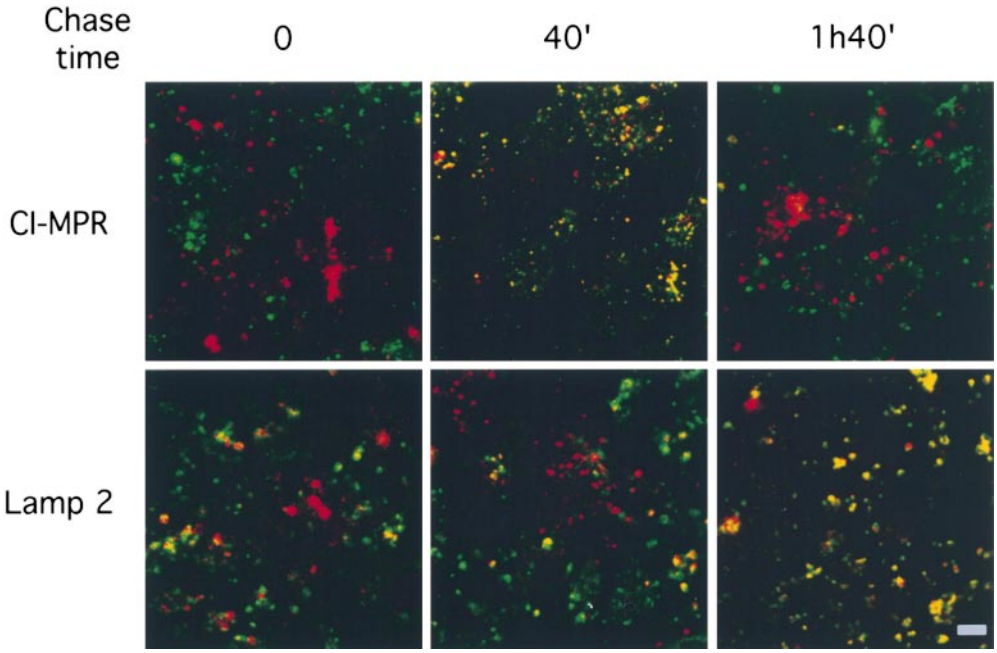

Figure 6. Colocalization of apically internalized LPS with markers for late endosomes and lysosomes. LPS was internalized at the apical surface of T84 cells for 20 min followed by no chase (0) or a chase in LPS-free medium for 40 min (40′) or 1 h and 40 min (1h40′). (Top) Anti–CI-MPR antibody was used to detect localization of LPS to late endosomal compartments. (Bottom) Anti–Lamp 2 antibody was used to localize LPS to lysosomal compartments. Only the apical plane is shown. Superimposed images revealed that at 40 min chase, LPS strongly colocalized with CI-MPR. At 1 h and 40 min chase, LPS predominantly localized to lysosomal compartments. Bar, 5 μm.

Pugin and Tobias, 1993). In addition, the structural composition of LPS may provide the means for specific interactions with a variety of membrane components including glycoproteins, phospholipids, and glycosphingolipids (Morrison, 1985). Evidence for LPS entry by receptor-mediated endocytosis provided in this study is purely morphological. Thus identification of an apical/brush border receptor remains a future prospect.

The routing of LPS in polarized epithelial cells after apical interaction is summarized in Fig. 7. Morphological studies identified the intracellular pathways that process LPS as consisting of peripheral compartments located just below the apical surface and in close proximity of the basolateral border directly below the intercellular tight junctions. By preloading endosomal compartments from the basolateral surface with transferrin, it was shown that apically internalized LPS was directly accessible, rapidly entering the same compartment. By intercepting the transferrin recycling pathway, this compartment provides LPS with an avenue to the basolateral side of the epithelium. This compartment was largely inaccessible to fluid-phase markers internalized at either the apical or basolateral pole. Complete colocalization of LPS with transferrin after 20 min of internalization indicated rapid entry of LPS into vesicles containing basolaterally applied transferrin but gave no indication of an intermediate compartment. The LPS transferrin-containing compartment may correspond to the apical transcytotic compartment described in the endocytic network of other cells (Hughson and Hopkins, 1990; Apodaca et al., 1994; Barroso and Sztul, 1994; Knight et al., 1995). The fact that the LPS transcytotic compartment corresponds to large multimembranous bodies suggests that the transcytotic pathway may be related to a maturation process of transcytotic vesicles from apical to basolateral. In contrast to what has been observed in the maturation process of the MHC class II (MIIC) compartment (Kleijmeer et al., 1997) and phagosomes (Desjardins, 1995), in which late phagosomes or multilamellar MIIC correspond to lysosomal-like compartments, late stages of transcytotic vesicle maturation would be characterized by the acquisition of molecules involved in the recycling pathway such as the transferrin receptor. The consequence of this specific property is the secretion of LPS in the lateral milieu.

After accessing transferrin positive basolateral endosomal compartments, electron microscopic analyses revealed exocytosis of LPS into the paracellular space beneath intercellular tight junctions. In addition, analysis of the fate of this molecule indicated that after apical internalization a significant amount of LPS was recycled back to and released at the apical surface. It is unclear as to whether recycling of LPS occurred from the apical transcytotic compartment or was an event that ensues from a transferrin-negative apical endosome before this compartment. Longer internalization revealed that LPS could be delivered to the late endosomal and lysosomal compartments as shown by colocalization of LPS with CI-MPR, rab 7, and Lamp. In addition, a 38% loss in the total amount of LPS as detected by ELISA and the LAL assay supported the contention that some LPS accessed lysosomal compartments and was processed. However, direct demonstration that LPS that reaches the late lysosomal compartments was actually detoxified and further processed remains to be shown. It is unclear, for instance, if a lysosomal preparation from T84 cells does contain an enzyme equivalent to the acyloxyacyl hydrolase of neutrophils required for detoxification of lipid A (Luchi and Munford, 1993). In addition, LPS extracted from lysosomal preparations of cells should be tested against crude LPS for proinflammatory potential.

Depending on the cell type analyzed, a number of molecules transcytose polarized cell layers, with this process occurring from the apical to basolateral plasma membrane (as shown for IgG; Rodewald and Abrahamson, 1980;

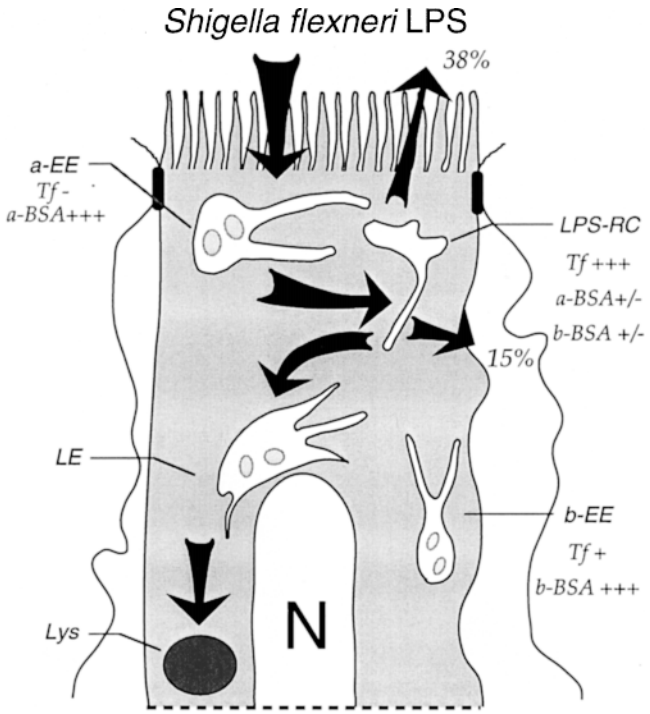

Figure 7. Model for intracellular trafficking of apically internalized LPS in polarized T84 cells. Apical LPS is internalized and after 20 min reaches a LPS-recycling compartment (LPS-RC). LPS-RC contains transferrin (Tf) and is poorly accessible to apically (a-BSA) and basolaterally (b-BSA) internalized BSA accumulated in apical (a-EE) and basolateral (b-EE) early endosomes, respectively. 38% of internalized LPS are recycled to the apical reservoir, 15% transcytosed and 47% targeted to late endosome (LE)/ lysosomal (Lys) compartments.

Rodewald and Kraehenbuhl, 1984), or from the basolateral to apical domain (IgA and IgA receptor; Stzul et al., 1983; Hoppe et al., 1985). IgA binds to polymeric immunoglobulin receptor at the basolateral surface and is transcytosed to the opposing surface being released in mucosal secretions as a first line of defense against lumenal threats. By inhibition of attachment and invasion, inactivation of toxins, opsonization, and complement activation, this factor of the humoral immune response neutralizes microbes at the apical epithelial surface of mucous membranes. Because IgA directed against a serotype-specific epitope of *S. flexneri* LPS has been proposed as an important element in the protection against shigellosis (Phalipon et al., 1995), this study has important implications on the role of IgA in intracellular neutralization of *Shigella* LPS. Analysis of the epithelial cell processing of microbial products may also provide information concerning intracellular interactions with epithelial histocompatibility antigens. Such interactions emphasize the complexity of the epithelium in the immunophysiology of the intestinal mucosa.

A question of considerable interest is the potential significance of nondetoxified LPS when allowed to transcytose the intestinal barrier. Transcytosed LPS may serve a physiological, as well as a pathological role. The physiological role of LPS may be restricted to the crypt area of the intestine. The crypt cells may channel a well-controlled quantity of LPS for two major purposes. (a) To maintain a low level of stress on the local innate immune system in order to hasten a nonspecific response to an infectious insult. (b) LPS may participate in intestinal differentiation. Previous studies have shown an inverse correlation between LPS transcytosis and epithelial cell differentiation with the most efficient passage of LPS occurring in cells of a state mimicking the less mature intestinal crypt cells (Beatty and Sansonetti, 1997). Expression of a putative apical receptor and/or access to a trafficking route may be arrested as cells differentiate on their way to the tip of the intestinal villi in the small intestine or to the higher part of the Lieberkühn crypts in the colonic and rectal mucosa. LPS may also have a pathological function if it is not properly contained. In inflammatory bowel diseases, initial inflammation is essentially in the intestinal crypts, indicating a potential role of some products of the intestinal flora in this region. In some infectious disease states such as shigellosis, initial inflammation also occurs in the crypts (Mathan and Mathan, 1986). It is possible that in certain serious conditions, the epithelial barrier to LPS is lifted, thus leading to an influx of LPS into the body with deleterious local and general effects (i.e., shock). The current study reflects the crucial aspect of the intestinal epithelium as a regulated barrier to LPS entry.

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