

Penetration of *Salmonella* Through a Polarized Madin–Darby Canine Kidney Epithelial Cell Monolayer

B. Brett Finlay,* Barry Gumbiner,§ and Stanley Falkow*

*Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305; and

§Department of Pharmacology, University of California, San Francisco, San Francisco, California 94143

Abstract. Many intracellular parasites are capable of penetrating host epithelial barriers. To study this process in more detail we examined the interactions between the pathogenic bacteria *Salmonella choleraesuis* and polarized epithelial monolayers of Madin–Darby canine kidney (MDCK) cells grown on membrane filters. Association of bacteria with the MDCK cell apical surface was an active event, requiring bacterial RNA and protein synthesis, and was blocked by low temperatures. *Salmonella* were internalized within a membrane-bound vacuole and exhibited penetration

through, but not between MDCK cells. A maximum of 14 *Salmonella* per MDCK cell crossed the monolayer per hour to the basolateral surface yet the monolayer remained viable and impermeable to *Escherichia coli*. Apical *S. choleraesuis* infection resulted in an increase in paracellular permeability but the MDCK intercellular contacts were not significantly disrupted. Basolateral *S. choleraesuis* infection was inefficient, and only small numbers of *S. choleraesuis* penetrated to the apical medium.

INTRACELLULAR parasites use a variety of routes to gain entry into susceptible hosts (Moulder, 1985). One such route is passage through the host's epithelial barrier, allowing the pathogen access to underlying tissue, blood, and the reticuloendothelial system. Pathogenic bacteria which are facultative intracellular parasites commonly use this method of entry and these include species of *Yersinia*, *Salmonella*, *Chlamydia*, *Shigella*, *Neisseria*, and *Brucellae*. The underlying mechanisms used by these microorganisms to penetrate host epithelia are not well understood. Part of the difficulty has been the lack of suitable in vitro methods to study epithelial cell penetration. Rather, most of our current knowledge has accumulated from animal studies.

A comprehensive descriptive study of *Salmonella* tissue penetration was published by Takeuchi (1967). Transmission electron microscopy was used to examine the intestinal epithelium of guinea pigs orally infected with *Salmonella typhimurium*. Takeuchi observed that as these bacteria came into close proximity to the brush border, the epithelial microvilli began to degenerate. Most of the bacteria entered directly into the epithelial cells and resided within membrane bound cavities. A few bacteria also appeared to enter at intercellular junctions and remained surrounded by a vacuole composed of neighboring epithelial cells. Neither bacterial exit from the epithelial cells nor entry into the underlying lamina propria was observed. Using murine ileal loops infected with *Salmonella typhi*, Kohbata et al. reported that ileal M cells, a type of intestinal epithelial cell, may be the target of *S. typhi* (1986). These authors found that M cells lost their microvilli, and suffered serious cytopathic effects. In vitro tissue

culture models have also been used to examine initial interactions and internalization of *S. typhimurium* and *S. typhi* into HeLa and HEp-2 cells (Giannella et al., 1973; Kihlstrom and Nilsson, 1977; Yabuuchi et al., 1986; Yokoyama et al., 1987). Internalization of *Salmonellae* into these cells appeared to proceed by a similar mechanism to that observed with the guinea pig model. We have demonstrated that *Salmonella* replicates inside vacuoles within epithelial cells, and bacterial entry and intracellular replication does not require endosome acidification (Finlay and Falkow, 1988).

In this study we have focused our attention on *Salmonella choleraesuis*, a common pathogen of swine which is also a highly invasive and serious human pathogen. Unlike most other nontyphoid *Salmonellae*, *S. choleraesuis* rarely causes gastrointestinal symptoms; instead it is considered the prototype of *Salmonella* which cause invasive diseases (Rubin and Weinstein, 1977). *S. choleraesuis* infections often lead to serious bacteremia, with a fatality rate 2–3-fold greater than typhoid fever and other clinical manifestations include osteomyelitis, meningitis and metastatic lesions.

To investigate the events that lead to *Salmonella* invasion, we examined the interaction of this pathogen with Madin–Darby canine kidney (MDCK) cells, a polarized epithelial cell line isolated from the kidney of a cocker spaniel (ATCC, 1981). When grown on permeable supports, these cells will form a polarized epithelial monolayer which is impermeable to ions, has a measurable transepithelial electrical resistance, and has several defined apical and basolateral surface markers (reviewed by Simons and Fuller, 1985). Physiologists have long used this system for studying epithelial transport

while cell biologists have used it to study cell polarity development and maintenance, and transcytosis (Simons and Fuller, 1985). Several workers have infected polarized monolayers with viruses and demonstrated that viral infections and maturation can be a polar event (Fuller et al., 1984). Bacterial infection of polarized cells has not been reported, however. In the present study we characterized the interactions which occur between *S. choleraesuis* and polarized MDCK cell monolayers. This experimental system permitted us to measure the initial interactions which occur between bacteria and host cells such as adherence and invasion, and led to the remarkable observation that this bacterium penetrated through viable MDCK monolayers.

Materials and Methods

Bacteria

Salmonella choleraesuis var. Kunzendorff strain 38₁ was kindly provided by N. Nnalue and B. Stocker of the Department of Medical Microbiology (Stanford University, Stanford, CA). This particular strain, designated SL2824 (Nnalue and Stocker, 1986), is mouse and pig virulent, has the somatic antigen composition O 6₃, 7 and is resistant to streptomycin. *Escherichia coli* strain DH5 α (F⁻, end A1, hsd R17 (r_K⁻, m_K⁺), supE44, thi-1, λ ⁻, rec A1, gyr A96, rel A1, ϕ 80d_{lacZ} Δ M15) was purchased from Bethesda Research Laboratories (Gaithersburg, MD). The vector pACYC184 (Cm^R, Tc^R; Chang and Cohen, 1978), was transformed into DH5 α to provide selective antibiotic markers.

Bacteria were heat inactivated by incubating at 100°C, 30 min. Formalin inactivation was performed by resuspending bacterial cultures in 0.5% formalized saline, incubating for 18 h, 37°C, washing twice in PBS, and resuspending in L-broth. Polymyxin B periplasmic extracts were made by resuspending cultures in PBS containing polymyxin B (1 mg/ml), shaking at 37°C, 1 h, and then filtering with a 0.2- μ m filter. Crude bacterial cell membranes were obtained by sonication of bacterial cultures and pelleting in an Eppendorf centrifuge.

MDCK Cells

Strain 1 MDCK cells were used between passage 18 and 50. Cells were grown in MEM, 10 mM Hepes, pH 7.3, and 5% FCS without antibiotics as described (Balcarova-Stander et al., 1984). Cells were passed twice weekly with a split ratio of 1:5. Transwell filter units (model No. 3415, Costar, Cambridge, MA) contained a 0.33-cm² porous filter membrane (3.0- μ m pores) that had been treated for tissue culture. Filter units were incubated in 24 well microtitre plates (Costar). These units were placed in MEM containing 10% FCS, penicillin (100 U/ml) and streptomycin (100 μ g/ml) for 30 min before seeding. Preincubation medium was removed and 150 μ l of a trypsinized MDCK cell suspension (1.5×10^5 cells) was added to each Transwell unit and placed in 1 ml fresh medium. Monolayers were used after 4 d incubation (37°C, 5% CO₂). After this time there were $\sim 3.5 \times 10^5$ MDCK cells/filter. Before bacterial addition monolayers were incubated in MEM containing 10% FCS without antibiotics for 1 h.

Monolayers were infected with bacteria by removing the medium in the Transwell unit and adding 5 μ l (3.5×10^6) freshly grown bacteria to the monolayer. Fresh medium was added to both surfaces and the units incubated.

Monolayer Association Assay

Bacteria in mid-log phase growth were washed once and resuspended in methionine assay medium (Difco; Detroit, MI). After a 30-min incubation (37°C), bacteria were centrifuged and resuspended in assay medium containing 50 μ Ci/ml [³⁵S]methionine (New England Nuclear, Boston, MA) and incubated for another 30 min. Bacteria were washed thrice in L-broth and 5 μ l was added to either surface of a polarized monolayer as described above. After appropriate incubation times monolayers were washed several times in cold PBS and the filter removed by excision and placed in 5 ml aqueous counting scintillant (ACS II; Amersham, Arlington Heights, IL). To examine the effects of bacterial protein synthesis inhibitors, bacteria were preincubated in chloramphenicol (30 μ g/ml) or gentamicin (100 μ g/ml) for 30 min before adding to the monolayer. These concentrations of antibiotics were maintained in the filter medium.

Quantitation of Bacterial Transcytosis

S. choleraesuis and *E. coli* DH5 α (3.5×10^6 bacteria each) were added together to either surface of polarized monolayers. These bacteria continued to divide approximately once every hour in this medium, until reaching a maximum density of 1×10^9 cells after 8 h. The medium opposite the side of bacterial addition was removed every hour and replaced with fresh 37°C medium. The number of bacteria in this medium was titered by plating appropriate dilutions on selective antibiotic plates, incubated, and viable counts determined.

Ca²⁺ Removal from Monolayers

Polarized monolayers were washed several times in PBS (no Ca²⁺ or Mg²⁺) and incubated in Ca²⁺ free minimal essential amino acids with spinner salts (SMEM, Gibco) and 10 mM Hepes, pH 7.4. Electrical resistance measurements were used to monitor disruption of cell junctions.

Electrical Resistance Measurements

Transmonolayer electrical resistance measurements of MDCK monolayers grown in Transwell units were performed with an apparatus similar to that described by Perkins and Handler (1981). Resistance was calculated from the change in voltage across the monolayer induced by short 4 μ A pulses of current. The area/resistance (Ω cm²) was calculated by multiplying the measured resistance by the area of the filter.

Transmission Electron Microscopy

MDCK monolayers grown in Transwell units were washed seven times with PBS and fixed in cold (4°C) 2% glutaraldehyde, 0.1 M sodium phosphate buffer (pH 7.4) overnight. After washing with phosphate buffer, samples were postfixed in cold 1% OsO₄ in 0.1 M phosphate buffer for 90 min, and then stained with cold 0.25% uranyl acetate overnight. Samples were dehydrated in a series of alcohols and embedded in a firm Spurr's plastic. Samples were sectioned and stained with uranyl acetate and lead citrate before examination in a Phillips 201c electron microscope.

Scanning Electron Microscopy

Monolayers were fixed in glutaraldehyde as described above. Samples were dehydrated in a critical point apparatus (Polaron) and, after a gold evaporation step, were examined with a Cambridge S 4 Stereoscan scanning electron microscope.

[³⁵S]Methionine-Uptake Measurements

This assay was performed similar to that described by Balcarova-Stander et al. (1984). Monolayers grown in Transwell units were infected with bacteria as described above. After appropriate incubation times monolayers were washed and then incubated for 30 min, 37°C in prewarmed low methionine medium (Eagle's minimal essential medium containing Hank's salts, 25 mM Hepes buffer, chloramphenicol [100 μ g/ml], tetracycline [25 μ g/ml], and gentamicin [100 μ g/ml]). These antibiotics inhibited bacterial methionine uptake by 98%. After preincubation, medium from either the basolateral or apical side was replaced with fresh medium containing [³⁵S]methionine and incubated for 5 min. Filter units were washed twice in cold medium, the filters were excised with a scalpel, and washed several more times. Filters were placed in 250 μ l 2% SDS in 10 mM Tris-HCl, pH 7.6, heated for 5 min, 100°C, and vortexed vigorously. 100- μ l aliquots were spotted in duplicate on Whatman 3-MM paper, TCA precipitated as described (Balcarova-Stander et al., 1984), and incorporated radioactivity counted by scintillation.

Immunofluorescence

Indirect immunofluorescence was performed on MDCK cell monolayers grown in Transwell units. Monolayers grown in these units were infected with bacteria and incubated for 4 h. After measuring electrical resistance monolayers were washed three times in PBS, fixed in -20°C methanol for 4 min, and rinsed in acetone (-20°C). Monolayers were then dried and rehydrated in PBS containing 0.2% gelatin (15 min). rr1 monoclonal antibodies were used to stain uvomorulin (Gumbiner and Simons, 1986). Texas-red conjugated anti-mouse antibodies (Accurate Chemicals, Westbury, NY) were used to stain rr1. Phalloidin (Molecular Probes, Eugene, OR), diluted 1 to 10 in PBS, was used to stain cellular actin. Hoechst stain was added

to visualize cell nuclei (Fuller et al., 1984). Filter units were placed on a 20- μ l drop of antibody, 30 μ l antibody solution was added to the apical surface, and the filter units were incubated 30 min, 23°C. Filter units were rinsed with PBS gelatin five times, 5 min per wash after each antibody staining. The filter was excised and embedded in moviol underneath a coverslip on a glass slide. Filters were viewed by fluorescence microscopy.

Invasion Assay

This assay was performed as described elsewhere (Finlay and Falkow, 1988). 5 μ l of standing overnight bacterial cultures were added to MDCK cell monolayers, and incubated for 2 h at 37°C in a 5% CO₂ atmosphere. Monolayers were washed three times with PBS and then incubated another 2 h in fresh medium containing 100 μ g/ml gentamicin. This treatment kills extracellular bacteria but does not affect viability of intracellular organisms. Monolayers were washed thrice with PBS and then lysed with a 1% Triton X-100 solution. Appropriate dilutions were spread onto L-agar plates and colony forming units were counted.

Results

Salmonella Association with Polarized MDCK Cell Monolayers Requires Bacterial Protein Synthesis and Causes Loss of Host Microvilli

The kinetics of bacterial association with monolayers were determined by adding ³⁵S-labeled Bacteria to either the apical or basolateral surface of a polarized MDCK monolayer. After appropriate incubation times, filters containing monolayers were extensively washed with PBS, excised from their plastic support, and counted in a liquid scintillation counter (see Materials and Methods). This method does not distinguish between bacteria which are strongly attached to the monolayer and those which have actually entered the MDCK cells. Nonadherent, noninvasive *E. coli* DH5 α did not associate with the monolayer when added to either the apical or basolateral surface (Fig. 1, Table I). 0.5% of the radiolabeled *S. choleraesuis* which were added to the basolateral (bottom) surface remained associated with the monolayer after 6 h. In contrast, 12% of *S. choleraesuis* added to the apical (top) surface adhered to or invaded the monolayer after 6 h (Fig. 1), indicating that *S. choleraesuis* may have a predilection for the apical surface of MDCK cell monolayers. *S. choleraesuis* began to associate with the apical surface after 2 h, and then the numbers of attached and invading bacteria steadily increased for the next 4 h.

We then attempted to dissociate bacterial adherence from bacterial internalization. It has been shown that another invasive Enterobacteriaceae, *Yersinia pseudotuberculosis*, adheres to HEP-2 monolayers at 4°C, but is not internalized until the temperature is raised (Isberg et al., 1987). Binding but

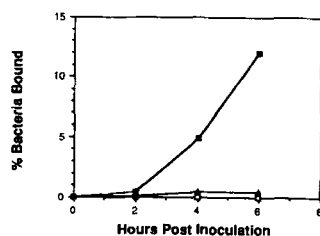


Figure 1. Association of bacteria with a polarized epithelial monolayer. ³⁵S-labeled bacteria were added to filter grown MDCK cells, incubated, washed, and monolayers counted as described in Materials and Methods. (% bacteria bound) Percent of the radiolabeled inoculum which remained associated with the monolayer after extensive washing. (□) Apically added *E. coli* DH5 α ; (▲) basolaterally added *S. choleraesuis*; (■) apically added *S. choleraesuis*.

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Table I. Effect of Temperature and Protein Synthesis Inhibitors on Salmonella-Monolayer Association

Bacteria added	Treatment	Associated* %
<i>S. choleraesuis</i> , apical	37°C	4.9
	4°C	0.08
	37°C, Cm	0.14
	37°C, Gent	0.16
<i>S. choleraesuis</i> , basolateral	37°C	0.47
	4°C	0.05
<i>E. coli</i> DH5 α , apical	37°C	0.05
	4°C	0.03

* Represents the percentage of counts of radiolabeled bacteria which remained associated with the monolayer after 4 h incubation. Cm, chloramphenicol; Gent, gentamicin.

not internalization at 4°C is also characteristic of molecules which are internalized by receptor mediated endocytosis (Willingham and Pastan, 1984). Unlike *Yersinia pseudotuberculosis*, *S. choleraesuis* does not associate with MDCK cell monolayers incubated on ice (Table I). Further evidence that the association between *S. choleraesuis* and MDCK monolayers was not a simple "receptor-ligand" association but instead an active process was obtained by using inhibitors of bacterial protein synthesis. Addition of chloramphenicol or gentamicin inhibited *S. choleraesuis*-monolayer association (Table I). Chloramphenicol is a bacteriostatic antibiotic and, in the presence of this drug, the bacteria retained viability (data not shown). Bacteria treated with gentamicin, a bactericidal drug, were not viable. Bacterial association with the monolayer was also completely inhibited when rifampin, a bacteriostatic drug which inhibits RNA synthesis, was present (data not shown).

Scanning electron microscopy revealed several features of bacterial infection of MDCK cells. As shown in Fig. 2 a, the apical surface of polarized MDCK cells contains many evenly distributed microvilli. Addition of *S. choleraesuis* to the apical surface caused individual MDCK cells to appear denuded within 1 h, yet neighboring cells appeared unaffected (Fig. 2 b). MDCK cells devoid of microvilli always had at least one bacterium associated with their surface. However, several bacteria could be observed close to normal appearing MDCK cells (Fig. 2 b). Micrographs of monolayers 4 h after adding bacteria revealed several *S. choleraesuis* in the process of entering MDCK cells (Fig. 2 c). Each bacterium was associated with an indentation in the MDCK cell surface. These "craters" varied in depth, with some nearly enclosing the bacteria. At later time points most MDCK cells were infected, and no microvilli were visible (data not shown).

Salmonella Penetration Through Polarized Monolayers

We grew MDCK cell monolayers on filters with 3- μ m pores to examine bacterial passage through this barrier. This system allowed us to quantitate the rate at which bacteria passed through a polarized monolayer. Noninvasive *E. coli* DH5 α were not capable of passing through these monolayers in either direction, even after 48 h incubation (data not shown). However, if epithelial cell tight junctions were disrupted by the addition of Ca⁺² free medium (SMEM, Materials and

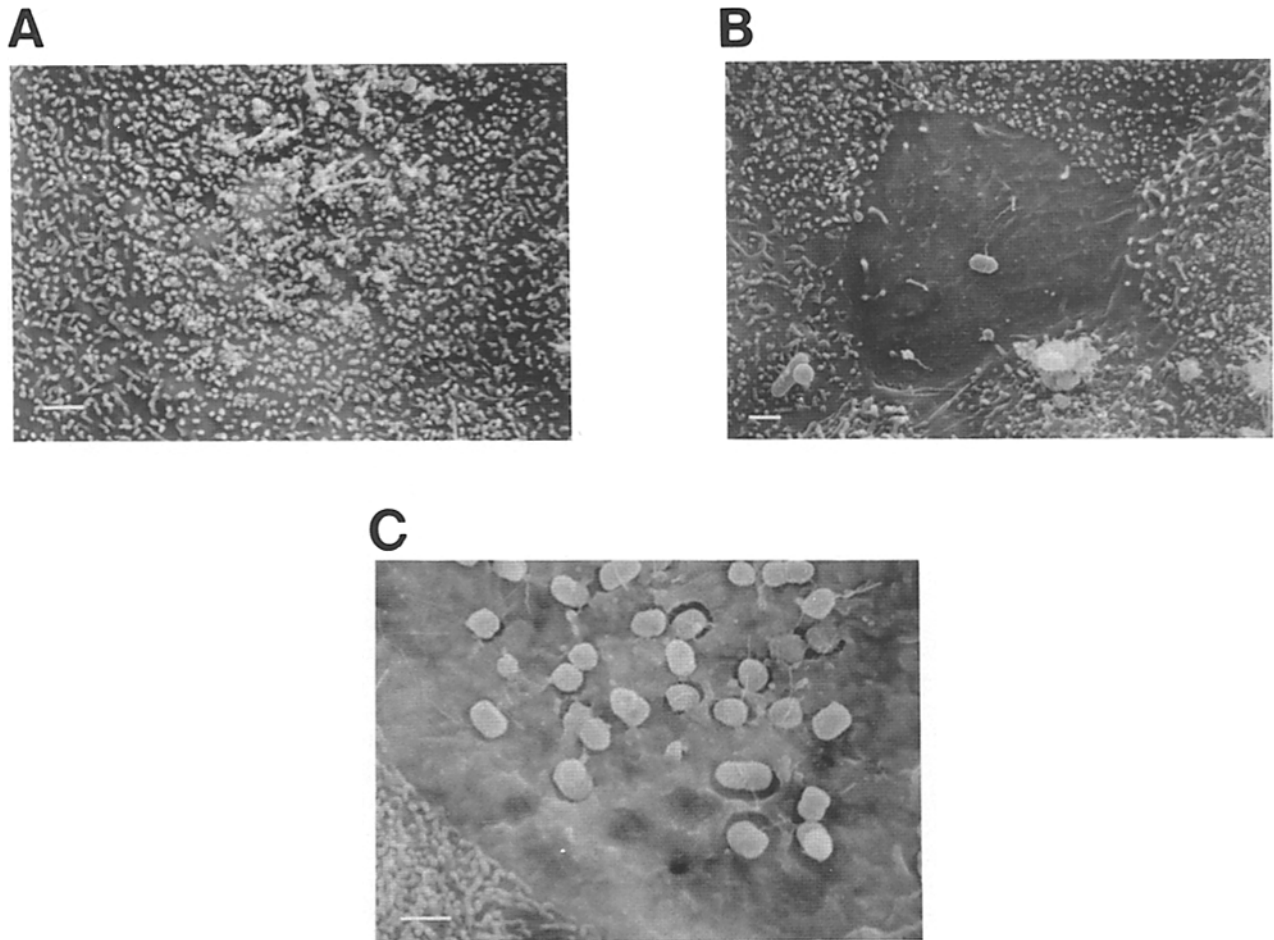


Figure 2. Scanning electron micrographs of the apical surface of polarized monolayers. (A) Uninfected monolayers exhibiting uniform cell microvilli. (B) Monolayer 2 h after addition of *S. choleraesuis* to the apical surface. Microvilli loss was observed on isolated MDCK cells. Central to the frame is an isolated bacterium with a visible flagellum. (C) MDCK cell surface 4 h after addition of *S. choleraesuis* to the apical surface. Indentations in the membrane are visible underlying bacteria. In the lower left corner is a neighboring MDCK cell which has retained its microvilli. Bars, 1 μ m.

Methods), these bacteria penetrated the filter immediately (data not shown). We therefore used *E. coli* DH5 α as a sensitive internal measure of monolayer integrity, as they allow detection of a small number of exposed pores in the monolayer.

Fig. 3 shows the quantitative measurement of *S. choleraesuis* passage through a polarized MDCK monolayer. The number of bacteria detected includes those which passed through the monolayer in 1 h as well as those which divided after reaching the opposite side. The generation time of *S. choleraesuis* in MEM media is \sim 50 min, while that of *E. coli* DH5 α is slower (data not shown). Therefore the numbers presented in Fig. 3 could, at most, be twice the number of bacteria actually passing through the filter. (We could not use bacteriostatic antibiotics to inhibit bacterial RNA and protein synthesis as these drugs inhibited bacterial invasion, as discussed previously. Attempts to use bacteriostatic drugs which inhibit bacterial DNA replication were also unsuccessful, but for different reasons. These agents did not inhibit initial interactions, but decreased the viability of the bacteria over time, producing deflated numbers of bacteria in the penetration assay.)

Neither the invading *Salmonella* nor the marker *E. coli* passed through the monolayers during the first 3 h, indicating that these monolayers were intact. After 4 h small numbers of *S. choleraesuis* could be found in the basolateral medium of monolayers infected from the apical surface (Fig. 3 a). The numbers of *S. choleraesuis* in the basolateral medium continued to increase until 9 h post infection, at which point \sim 5 \times 10⁶ bacteria had penetrated 3.5 \times 10⁵ MDCK cells per hour, a rate of 14 bacteria/MDCK cell per hour. After 10 h *E. coli* DH5 α were present in the basolateral medium indicating loss of monolayer integrity. (The difference in numbers between these two species at the 15 h time point is possibly due to the slower generation time and also slower motility of *E. coli* DH5 α .)

Addition of bacteria to the basolateral surface revealed different penetration kinetics (Fig. 3 b). Although equal numbers of bacteria were used to infect both surfaces, far fewer bacteria penetrated the monolayer from the basolateral surface. No *S. choleraesuis* were observed in the apical medium until 8 h after bacterial addition. Basolaterally added *S. choleraesuis* continued to penetrate until reaching a constant rate of \sim 5 \times 10⁴ bacteria per hour at 13 h post

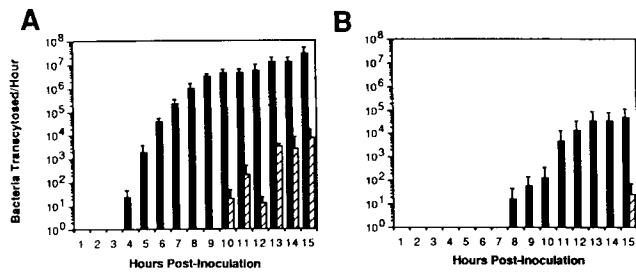


Figure 3. Quantitation of bacterial penetration. Equal amounts (3.5×10^6 bacteria) of *S. choleraesuis* (solid bars) and *E. coli* DH5 α (hatched bars) were added together to either the apical (A) or basolateral (B) surface and the numbers of bacteria/hour present after crossing the monolayer were quantitated (Materials and Methods).

infection (0.14 bacteria/MDCK cell per hour). Non-invasive *E. coli* DH5 α first appeared in the apical medium after 15 h.

Trypan blue is a dye which is excluded from viable eucaryotic cells, but stains dead cells and is used for determining cell viability (Wilson, 1986). As illustrated in Fig. 4, MDCK cell viability does not decrease until at least 8 h after addition of *S. choleraesuis* to the apical surface as determined by trypan blue exclusion. No decrease in viability was observed with basolaterally infected monolayers (Fig. 4), *E. coli* DH5 α , or uninfected monolayers (data not shown). Loss of viability 8 h after apical addition correlates with leakage of *E. coli* DH5 α to the basolateral surface between 9 and 10 h post infection (Fig. 3 a). Further evidence indicating that MDCK cells are viable while *S. choleraesuis* penetrates and passes through the monolayer was obtained from [35 S]methionine uptake studies (see below). Taken collectively these results indicate that greater than 10^6 apically added *S. choleraesuis* can pass through a viable MDCK monolayer per hour.

We undertook a comprehensive study of *S. choleraesuis* penetration of MDCK cell monolayers by using transmission electron microscopy. 30 min after bacterial addition to the apical side very few *S. choleraesuis* were in contact with the monolayer surface, although small numbers of bacteria were present within membrane bound vacuoles inside MDCK cells. Fig. 5, a-c is representative of the typical sequence of events involved in *S. choleraesuis* internalization into a MDCK cell. Initially the bacterium makes contact with the host cell surface, often involving microvilli (Fig. 5 a). These microvilli soon disappear, as previously mentioned. A cavity or indentation of the host cell membrane forms under the bacterium, and the bacterium is internalized (Fig. 5 b). Coated pits were often, but not always observed in the host cell membrane in close proximity to the bacterium. Invaginations containing bacteria then closed, usually leaving a single organism surrounded by a host membrane (Fig. 5 c). Bacteria remained inside membrane bound vacuoles, often dividing intracellularly in these enclosures (Finlay and Falkow, 1988). Between 2 and 4 h after addition of bacteria increasing numbers of *S. choleraesuis* interacted with the surface of host cells (Fig. 5, d and e) in agreement with the monolayer association data. At later time points several vacuoles containing bacteria were visible inside most MDCK cells (Fig. 5, d and e), with one or two bacteria within each vacuole (4-8

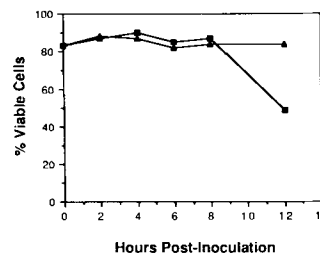


Figure 4. Trypan viability of *S. choleraesuis* infected monolayers. Monolayers were infected and incubated as described (Materials and Methods). After washing in PBS, monolayers were incubated in 30 μ l 0.05% trypsin, 0.02% EDTA in PBS, 37°C, 10 min. 150 μ l of trypan blue dye (0.16% in PBS) was added and viable and nonviable cells were counted by light microscopy. >500 cells were examined for each point. (\blacktriangle) *S. choleraesuis* added to the basolateral surface; (\blacksquare) *S. choleraesuis* added to the apical surface.

h). No bacteria were ever observed in the intercellular regions between MDCK cells. We were unable to observe any bacteria in the process of exiting the host cell.

Penetration but not Invasion of *S. choleraesuis* is Inhibited by Incubation at Temperatures Less Than 37°C

We tested the rates of penetration of *S. choleraesuis* across MDCK cell monolayers at various temperatures to determine if bacterial penetration was affected. As illustrated in Table II, incubation at either 23° or 28°C completely abolished passage of *S. choleraesuis* across polarized monolayers. However, invasion of monolayers grown on plastic supports was lowered only slightly at these temperatures, as were bacterial growth rates. This is in contrast to the lack of inhibition of transcytosis of the vesicular stomatitis virus G protein seen at 20°C in polarized MDCK cells (Pesonen et al., 1984).

***S. choleraesuis* Causes Loss of MDCK Monolayer Resistance and Methionine Uptake Polarity but does not Significantly Alter Intercellular Junctions**

Development of a MDCK monolayer into sealed epithelial barriers is reflected by measurement of electrical resistance across the monolayer. Fully confluent strain I MDCK cells often exhibit resistances greater than 3,000 Ω cm 2 on 0.45- μ m pore filters; this resistance is slightly lower when grown on 3- μ m pores (Balcarova-Stander et al., 1984; Fuller et al., 1984). Resistance is largely a measure of the integrity of tight junctions between the monolayer's cells (Gumbiner and Simons, 1986). We conducted electrical resistance measurements over the course of *Salmonella* infection of MDCK cell monolayers. These measurements also allowed us to determine if *S. choleraesuis* affects tight junctions between epithelial cells. Initial electrical measurements of uninfected MDCK cell monolayers indicated that almost every monolayer had an electrical resistance of $\sim 1,100 \pm 100 \Omega$ cm 2 (Fig. 6). This resistance was $600 \pm 75 \Omega$ cm 2 after 2 h, and remained at this level for at least 12 h (Fig. 6). We attributed this initial resistance drop to handling procedures during this time, as this drop was observed in all samples tested. This resistance is lower than that achieved by using 0.45 μ m pores, but still indicates the presence of a highly impermeable monolayer.

The addition of *E. coli* DH5 α to either the apical or basolateral monolayer surface had no effect on electrical resistance even after 12 h incubation (data not shown). Basolateral infection with *S. choleraesuis* also had no effect

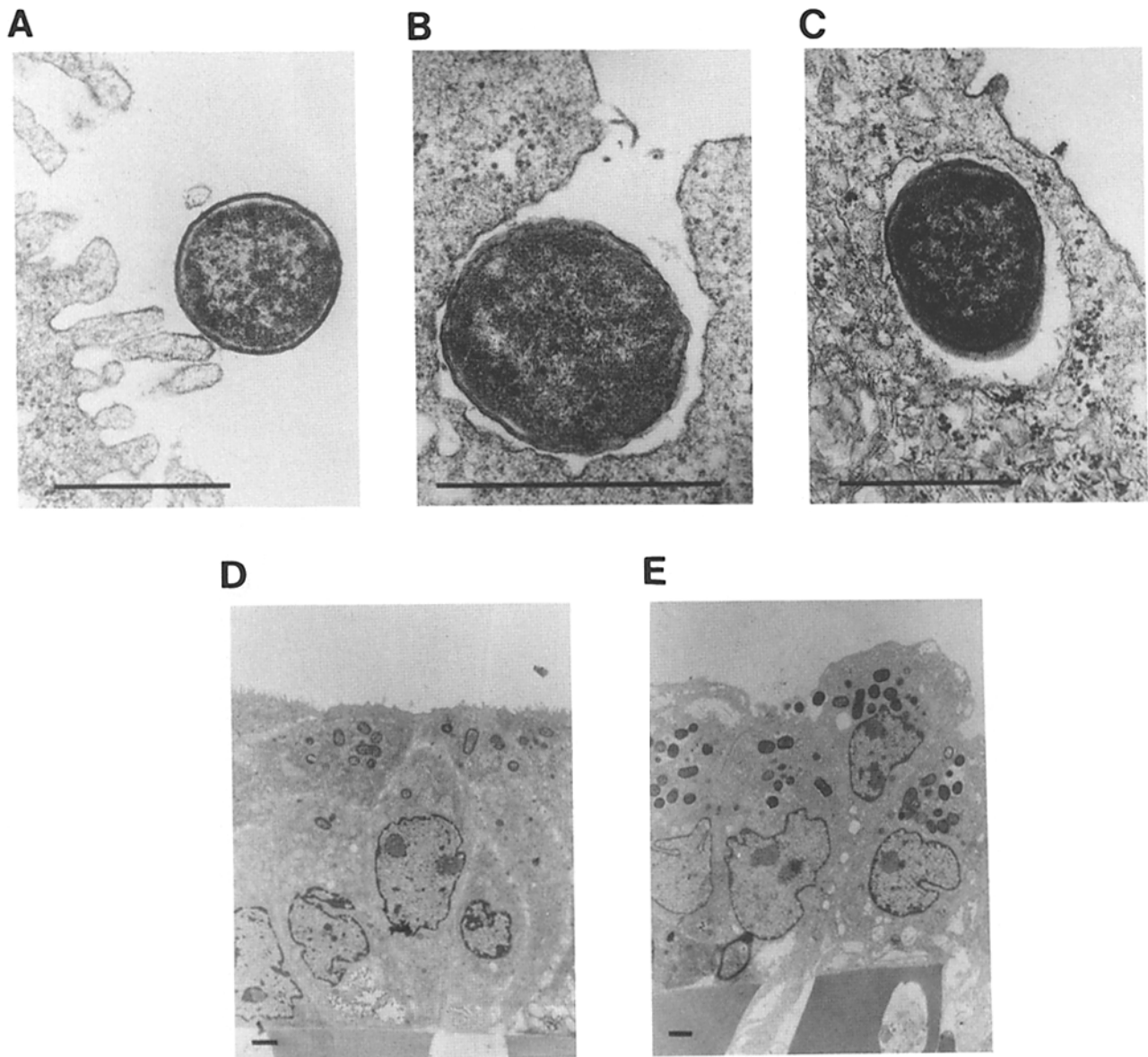


Figure 5. Transmission electron micrographs of MDCK cell monolayers infected from the apical surface with *S. choleraesuis*. Samples were prepared as described in Materials and Methods. (A) *S. choleraesuis* interacting with host microvilli, 2 h post infection. (B) *S. choleraesuis* being internalized into an MDCK cell 4 h post infection. (C) *S. choleraesuis* internalized within a vacuole, 4 h post infection. (D) Polarized monolayers grown on filters with 3- μ m pores, 2 h after bacterial addition. Microvilli are still present on some cells. (E) Polarized monolayers 6 h after bacterial infection. Microvilli are not apparent and bacterial invasion is uniform throughout the monolayer. Bars, 1 μ m.

on resistance, exhibiting a resistance curve nearly identical to uninfected monolayers (Fig. 6). However, addition of *S. choleraesuis* to the apical surface caused a partial loss of resistance by 2 h and a complete loss after 4 h (Fig. 6), yet the MDCK cells were viable for at least another 4 h after loss of electrical resistance (Fig. 4). This resistance loss was dependent on the number of *S. choleraesuis* present, as addition of 100-fold fewer bacteria resulted in complete resistance loss after 6 h, while 10,000-fold fewer bacteria took \sim 10 h before electrical resistance was eliminated (data not shown). Another common pathogenic *Salmonella* species, *S. enteritidis*, also caused a complete drop in resistance within 4 h when added to the apical surface and was transcytosed at rates similar to *S. choleraesuis* (data not shown).

Table II. Effect of Temperature on *S. choleraesuis* Penetration and Invasion

Temperature	Bacteria invaded*	No. bacteria penetrated†
$^{\circ}$ C	%	
23 $^{\circ}$	1.7	0
28 $^{\circ}$	1.4	0
37 $^{\circ}$	3.5	3.0×10^5

* Values represent the percent of the initial bacterial inoculum which was viable after 2 h incubation with a monolayer (2×10^5 MDCK cells) followed by 2 h gentamicin treatment (100 μ g/ml). This assay is described in Materials and Methods and elsewhere (Finlay and Falkow, 1988). Values represent the average for two samples.

† Values are the number of bacteria which penetrated across a polarized monolayer per hour, after 7 h incubation at the appropriate temperature. Duplicate monolayers were infected as described in Materials and Methods.

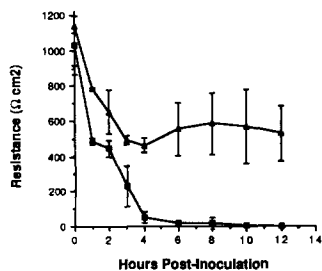


Figure 6. Electrical resistance measurements across infected epithelial monolayers. Resistances were measured on monolayers as described (Materials and Methods). (▲) *S. choleraesuis* added to the basolateral surface; (■) *S. choleraesuis* added to the apical surface. Measurements are an average of five filters \pm SD. Resistance measurements of monolayers infected with *E. coli* DH5 α were the same as uninfected monolayers (data not shown).

Measurements are an average of five filters \pm SD. Resistance measurements of monolayers infected with *E. coli* DH5 α were the same as uninfected monolayers (data not shown).

The factor(s) which produce this resistance drop was dependent upon the presence of viable bacteria. 0.2- μ m filtrates of broth in which *S. choleraesuis* had been grown had no effect on resistance. *S. choleraesuis* membrane fractions, polymixin B elicited periplasmic extracts, or cytoplasmic extracts did not cause a drop in resistance; nor did formalin or heat killed bacteria (data not shown), indicating that it is an active bacterial process which produces monolayer resistance loss.

It has been reported that removal of Ca²⁺ from the medium abolishes electrical resistance by disrupting tight junctions (Gumbiner and Simons, 1986). We used Ca²⁺ free medium (SMEM) to compare loss of electrical resistance caused by Ca²⁺ removal and addition of *S. choleraesuis* to the apical surface. Incubation of polarized monolayers in SMEM for 1.5 h completely eliminated monolayer resistance (data not shown). *E. coli* DH5 α added to either surface of these monolayers were present in the opposite medium within 30 min of Ca²⁺ removal. This contrasts the results observed when *S. choleraesuis* is added to the apical surface, where electrical resistance is completely eliminated by 4 h, yet *E. coli* DH5 α do not penetrate for an additional 6 h after resistance loss.

Balcarova-Stander et al. (1984) have demonstrated that methionine uptake by polarized MDCK cell monolayers is localized to the basolateral surface. The extent of development of cell surface polarity can be measured by examining apical and basolateral uptake of [³⁵S]methionine. These workers reported that a basolateral to apical ratio of methionine uptake as high as 30 was routinely obtained with fully polarized cells. We decided to use this assay as a method for measuring the effect of *S. choleraesuis* on MDCK cell polarity.

Uninfected MDCK cell monolayers had a basolateral to apical methionine uptake ratio of \sim 16 (Fig. 7), indicating that MDCK monolayers grown in this manner are highly polarized. To successfully use this [³⁵S]methionine uptake assay with infected monolayers, bacterial protein synthesis needed to be inhibited. Preincubation in methionine-depleted medium which contained chloramphenicol, tetracycline, and gentamicin for 30 min before radionucleotide addition, but after appropriate bacterial infection times, inhibited bacterial incorporation of [³⁵S]methionine by 98% (Materials and Methods). Using this method we found that the basolateral to apical ratio of methionine uptake remained unaltered in the presence of *E. coli* DH5 α . Furthermore,

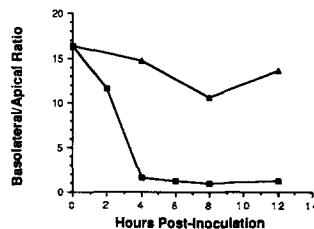


Figure 7. [³⁵S]Methionine uptake polarity of infected monolayers. Methionine uptake was measured for both apical and basolateral surface (Materials and Methods) and the basolateral:apical ratio calculated. (■) *S. choleraesuis* added to the apical surface; (▲) *S. choleraesuis* added to the basolateral surface. Uninfected monolayers and monolayers infected with *E. coli* DH5 α showed similar results to *S. choleraesuis* added to the basolateral surface (data not shown).

basolateral addition of *S. choleraesuis* did not alter this ratio (Fig. 7). However, by 4 h after addition of *S. choleraesuis* to the apical surface, polarity of methionine uptake was eliminated (Fig. 7), after which time this ratio remained at \sim 1.0. This loss completely parallels the loss of electrical resistance.

[³⁵S]methionine uptake is also a measure of cell viability and is commonly used to measure the effects of toxins on eucaryotic cells (Cawley et al., 1980). We found that the total amount of [³⁵S]methionine uptake was relatively constant over 8 h in monolayers apically infected with *S. choleraesuis*, and this uptake decreased slightly after 12 h (data not shown). These measurements correlate well with the trypan blue viability data indicating that MDCK cells are still viable up to 8 h post infection.

Several reagents exist which can be used to visualize cell junctions by immunofluorescence. Loss of intercellular contacts caused by decreasing calcium concentrations can often be detected by the redistribution of molecules involved in the formation of cell junctions from the normal sites of cell-cell contact. We used a monoclonal antibody (rr1) directed against an ovomorulin-like molecule that is associated with cell adhesion sites to examine the morphology of MDCK cell boundaries after addition of bacteria (Gumbiner and Simons, 1986). Monolayers which exhibited high resistance had distinct perimeters around individual MDCK cells (Fig. 8 a). These included monolayers infected with *E. coli* DH5 α and *S. choleraesuis* from the basolateral surface. We could observe no difference between these cells and monolayers which had been apically infected with *S. choleraesuis* and had lost their electrical resistance (Fig. 8 b). Fluorescence staining of monolayers treated with phalloidin, a stain for actin filaments, showed that the circumferential belt of actin filaments associated with cell junctions was not noticeably disrupted (data not shown). Examination of monolayers incubated in Ca²⁺ depleted medium exhibited obvious morphological differences with both stains (data not shown). These results again suggest that although apically added *S. choleraesuis* can eliminate transepithelial resistance, intercellular morphology is not grossly altered.

Discussion

We have shown that polarized MDCK cell monolayers can be used to study the interactions that occur between invasive *Salmonella* and epithelial cells. This in vitro system is similar in many respects to animal infection models. Loss of intestinal epithelial microvilli after *Salmonella* infection oc-

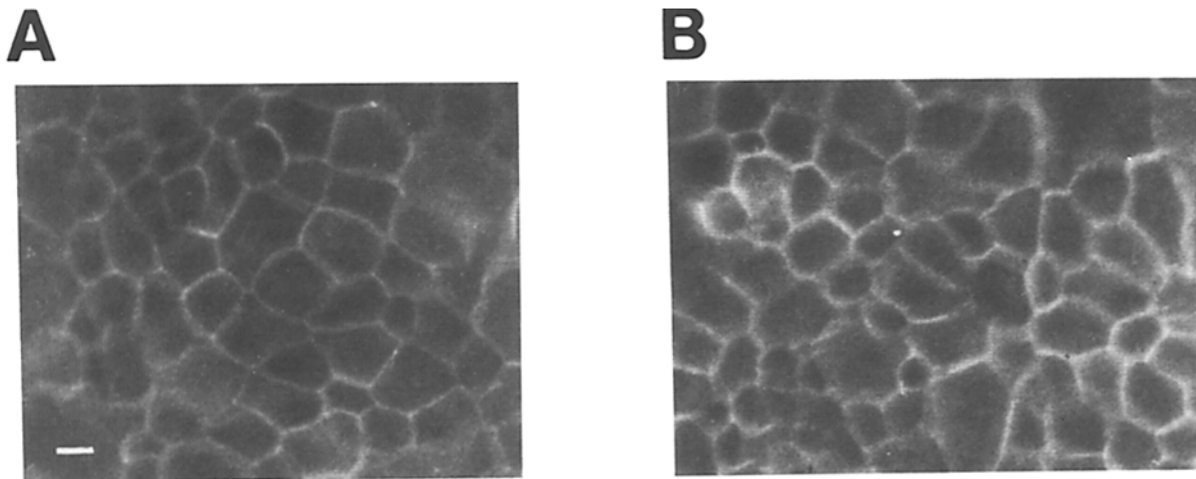


Figure 8. Indirect immunofluorescence of infected MDCK cells 4 h after addition of bacteria. Monolayers were permeabilized with methanol, and treated with the primary antibody rrl, a monoclonal which reacts with an uvomorulin-like polypeptide associated with cell junctions (Gumbiner and Simons, 1986). (A) Addition of *E. coli* DH5 α to the apical surface. This monolayer had a high electrical resistance. (B) Addition of *S. choleraesuis* to the apical surface. This monolayer exhibited no electrical resistance. Bar, 10 μ m.

curs in mice ileal loops (*S. typhi*; Kohbata et al., 1986) and in the guinea pig ileum (*S. typhimurium*; Takeuchi, 1967). Takeuchi reported uniform penetration of the guinea pig intestinal epithelium by *S. typhimurium*. These bacteria were internalized within membrane bound inclusions and remained within these vacuoles. After 24 h bacteria were observed within phagocytes in the underlying lamina propria indicating epithelial penetration. Apical *S. choleraesuis* infection of polarized MDCK monolayers demonstrated all of these qualities, making MDCK cells a very attractive in vitro model to study the precise mechanisms of *Salmonella* penetration of epithelial cells.

In vivo, the first intestinal epithelial surface encountered by ingested *Salmonella* is the apical surface. Only later in infections are *Salmonella* observed near the basolateral surface of epithelial cells, often in the underlying lamina propria (Takeuchi, 1967). Perhaps *Salmonella* interact predominantly with apical surfaces or a receptor located primarily on the apical surface, producing a directionality during infection in vivo. Vesicular stomatitis virus infects and buds from the basolateral surface of polarized MDCK cell monolayers (Fuller et al., 1984). In contrast, avian influenza viruses infect either surface and bud from the apical side (Fuller et al., 1984). We examined bacterial interactions with these monolayers and found that 10-fold more *S. choleraesuis* associated with the apical surface than the basolateral surface after 4 h of infection (Table I). After 6 h this ratio increased to 23 (Fig. 1). Bacterial penetration was also 100-fold greater using apically infected cells (Fig. 3). Although it is tempting to conclude that *S. choleraesuis* interacts predominantly with the apical surface of polarized monolayers, one should be cautious since the geometry of the filter is such that only a fraction of the basolateral surface may be accessible to bacterial interactions. (~15% of the filter consists of pores.) This area differential may contribute to the differences in surface association preference. Another possibility we considered was the effects of gravity on this system. However, *S. choleraesuis* is extremely motile, and centrifugation

at 15,000 g for 30 min did not pellet these bacteria, nor was there any difference in the rates of *Salmonella* passage in either direction through a filter with no MDCK cells, indicating that the effects of gravity on *S. choleraesuis* are minimal.

MDCK cell monolayers lost their transepithelial resistance by 4 h when *S. choleraesuis* was added to the apical surface. We examined whether *S. choleraesuis* may penetrate by disrupting the tight junctions and then passing between epithelial cells or by lysing the monolayer. Several lines of evidence rule this out. Electron microscopy indicated that MDCK cells were intimately associated, even after several hours of infection (Fig. 5) and bacteria were never observed between MDCK cells. Secondly, *S. choleraesuis* was able to pass from the basolateral to apical surface without necessarily altering cell surface polarity (Figs. 6 and 7). Motile *E. coli* DH5 α , which penetrated filters in the absence of a monolayer very rapidly (<30 min), did not enter the opposite medium for 6 h after loss of electrical resistance and methionine uptake polarity (Fig. 3). Removal of Ca²⁺, which disrupts tight junctions, allowed *E. coli* DH5 α to cross monolayers within 30 min. Immunofluorescence also indicated that cell-cell contacts were not significantly altered (Fig. 8). Taken collectively, these results suggest that *S. choleraesuis* passes through MDCK cells, rather than between them or by lysing the monolayer.

Transcytosis is a term used to describe transport of macromolecules (and their receptors) from one surface of a cell through the cell to the opposite surface (Mostov and Simister, 1985; Simons and Fuller, 1985). Transport of immunoglobulins (IgA and IgM) across epithelia are perhaps the best characterized examples of transcytosis (Mostov and Simister, 1985). Once these molecules interact with specific receptors on the cell surface, they are internalized by receptor mediated endocytosis and transported across the cell (Mostov and Simister, 1985). We have demonstrated that *S. choleraesuis* added to the apical surface of MDCK cells is capable of entering these cells, passing through these cells in a vacuole and escaping to the opposite medium. We consider this pro-

cess as a form of transcytosis since the bacteria pass through an impermeable epithelial monolayer to the opposite surface while the MDCK cells remain viable.

Up to 5×10^6 *S. choleraesuis* penetrate per hour through $\sim 3.5 \times 10^5$ MDCK cells, a rate of 14 bacteria/MDCK cell per hour. This process required a minimum of four hours, and reaches a maximum rate after 8 h. As illustrated in Fig. 1, $\sim 5\%$ of the initial inoculum is either adherent to or internalized within the MDCK cell monolayer 4 h after infection. Data from Table II indicates that 3.5% of the inoculum is internalized within MDCK cells at this time. These data suggest that at this time 70% of the bacteria which associate with the monolayer are internalized, while 30% (or 1.5% of the initial inoculum) are susceptible to gentamicin treatment. The number of bacteria penetrating the monolayer after 4 h represents 0.003% of the initial inoculum (Fig. 3) or 0.08% of the internalized bacteria. However, 6 h after infection this number rises to 2.8% of the initial inoculum while the percentage of labeled bacteria associated with the monolayer after 6 h is 12% (Fig. 1), indicating a significant increase in the number of bacteria penetrating the monolayer.

Transcytosis of IgA has also been studied in MDCK cells (Mostov and Deitcher, 1986). These workers bound labeled IgA to the basolateral surface at 4°C, after which monolayers were rapidly warmed and transcytosis quantitated. This process had a $t_{1/2}$ of 30 min, and was nearly complete after 2 h. Bacterial penetration appears slower than that of the IgA ligand, but, since *S. choleraesuis* did not associate significantly to 4°C monolayers, we were unable to uncouple binding and penetration rates and cannot determine whether this lag is due to initial binding, or due to differences in the mechanisms used by bacteria and simple ligands to cross a monolayer.

MDCK cells exhibit several morphological changes during *Salmonella* infection. These changes were observed only when *S. choleraesuis* was added to the apical surface. *Salmonella*-infected cells lost their microvilli, a phenomenon which has been reported in other infection models (Kohbata et al., 1986; Takeuchi, 1967). Perhaps *Salmonella* invasion causes depolymerization of microfilaments which support microvilli. Affecting microfilament polymerization could also contribute to the loss in electrical resistance and methionine uptake polarity, since actin filaments are involved in maintaining tight junctions (Meza et al., 1980). We used immunofluorescence of phalloidin stained monolayers to attempt to visualize differences in actin localization at cell junctions but did not detect any noticeable differences using this technique. Loss of transepithelial electrical resistance in MDCK monolayers occurs after infection with vesicular stomatitis and influenza viruses (Lopez-Vancell et al., 1984). 5–6 h after VSV infection an increase in transepithelial conductance (or loss of resistance) was observed, and was coincident with accumulation of envelope protein in the MDCK cell surface and viral budding, a basolateral phenomenon. Influenza virus, an apically budding virus, did not cause a drop in resistance until late (12–14 h) in the infection. This change was due to an effect on occluding junctions rather than membrane permeability (Lopez-Vancell et al., 1984). It was suggested that the loss in electrical resistance caused by VSV infection may be due to an actin depolymerizing effect, similar to that discussed above.

Both *Salmonella* and another invasive Enterobacteriaceae, *Yersinia*, infect hosts by penetrating the intestinal epithelium, and eventually enter the reticuloendothelial system. It was thought that similar invasive mechanisms may have been used by members of both families. Evidence presented here indicates that this is probably not true. In contrast to *Salmonella*, invasive *Yersinia* adhere to the eucaryotic cell surface, even on ice (Isberg et al., 1987). *Yersinia* are “embraced” by host microvilli which encircle the bacterium (Brunius and Bolin, 1983), and the organism is internalized within a membrane bound vacuole (Bovallius and Nilsson, 1975). Internalization of *S. choleraesuis* was associated with an indentation in the host cell surface underlying the bacterium, and entrapment of *Salmonella* by microvilli were never observed. Formalin and UV treated *Yersinia* appear to enter eukaryotic cells (Pedersen et al., 1979), again in contrast to our findings with *Salmonella*. It appears that *S. choleraesuis* plays a more active role in its internalization than *Yersinia*, and is not simply a passive “ligand” internalized by endocytosis.

Infection of polarized epithelial cells permits the exploration of several new facets of microbial pathogenesis. Since *S. choleraesuis* is a close relative of *E. coli* DH5 α , genetic manipulation of this organism is relatively easy. This system should allow us to identify and characterize genes responsible for the biological phenomena reported here. Besides examining invasion, we are now able to study previously unaddressed events involved in bacterial penetration from both the bacterial and eukaryotic perspective. This system may contribute to our limited understanding of the mechanisms involved in intracellular transport and transcytosis. Such model systems could be used to study other organisms which are known to cross epithelial barriers, a process fundamental to many human pathogens.

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