Host Restriction Phenotypes of *Salmonella typhi* and *Salmonella gallinarum*

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Salmonella typhi and *Salmonella gallinarum* phenotypes correlated with mouse host restriction have been identified by using in vitro and in vivo systems. *S. typhi* is capable of entering the murine intestinal epithelium via M cells, as is *Salmonella typhimurium*, which causes systemic infection in the mouse. But, unlike *S. typhimurium*, *S. typhi* does not destroy the epithelium and is cleared from the Peyer's patches soon after M-cell entry. *S. gallinarum* appears to be incapable of entering the murine Peyer's patch epithelium. Our in vitro evidence suggests that *S. gallinarum* is taken up in murine phagocytic cells by a mechanism different from that of *S. typhimurium*. *S. typhimurium* is taken up at a higher frequency and is maintained at higher viable counts throughout a 24-h time course in a murine macrophage-like cell line than are *S. gallinarum* and *S. typhi*.

Salmonella typhi, the etiologic agent of typhoid fever, infects humans and some nonhuman primates exclusively and is incapable of establishing infection in laboratory animals (8). *Salmonella gallinarum*, the causative agent of fowl typhoid, is restricted to avian hosts although it establishes a transient infection in laboratory mice and rarely causes food poisoning in humans (2). The genetic and molecular basis of host specificity exhibited by these salmonellae is not clear. In contrast, *Salmonella typhimurium* and *Salmonella enteritidis* can infect a variety of avian and mammalian hosts, including humans, with different infection outcomes ranging from gastroenteritis to bacteremia (16).

The best-studied model of human enteric fever is murine typhoid caused by *S. typhimurium*. The first step in the pathogenesis of murine typhoid has been shown to be entry into the murine M cells (5, 18). These specialized epithelial cells together with enterocytes line the domes of Peyer's patches in the intestine (17). *S. typhimurium* entry into cultured cells and murine M cells is associated with dramatic localized cytoskeletal rearrangement on the host cell surface, resulting in macropinocytosis and triggering of host-specified signal transduction cascades (3, 5, 11–13, 18). Subsequent to M-cell entry, *S. typhimurium* destroys the epithelial layer and invades mononuclear and probably lymphoid cells that lie under the infected M cells (18). The survival of *S. typhimurium* within cultured macrophages is consistent with the idea that phagocytic cells carry the infection from the Peyer's patches and adjacent mesenteric lymph nodes to the liver and spleen, where bacterial proliferation occurs in susceptible mice (4, 6).

In order to address the basis of this host specificity, we have focused on early events in infection. We assumed that the early steps of infection by *S. typhi* and *S. gallinarum* in their respective hosts are analogous to the early steps of infection by *S. typhimurium* in the mouse. Consequently, we investigated the capacity of these microorganisms to interact with the murine M cell and to interact with phagocytic cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. typhimurium* SL1344 (*hisG rpsL xyl*) (14) was provided by Bruce Stocker; *S. typhi* ISP1820 was provided by David Hone (15); *S. typhi* IAL, a clinical isolate from a patient in Brazil, was provided by Lee Riley; *S. typhi* 200Ty was provided by Bruce Stocker (9); *S. gallinarum* 91-29326 was provided by Ames, Iowa; and *S. gallinarum* 2933 was provided by Bruce Stocker. All strains of a given species behaved similarly in these experiments. A laboratory stock of *Escherichia coli* HB101 was used. Bacteria to be assayed for invasiveness in vivo or for entry into and/or growth in macrophage-like cell lines were grown as standing overnight cultures, a growth condition that was shown to confer increased invasiveness to various *Salmonella* strains (20). Cultures were started from frozen aliquots or individual colonies by growing them for 6 h on a roller drum (80 rpm) (New Brunswick Scientific, New Brunswick, N.J.) at 37°C. Then, the cultures were diluted 1:1,000 into 3 to 5 ml of Luria-Bertani (LB) broth in a Pyrex no. 9820 tube (1 by 10 cm) and grown standing at 37°C for 10 to 14 h.

Tissue culture conditions and invasion assay. RAW264.7 cells (ATCC TIB 71) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, Md.) containing 10% fetal bovine serum (Gibco BRL) and passaged every 2 to 3 days.

The intracellular growth of the various *Salmonella* strains was monitored by a gentamicin resistance assay (10). Macrophages (2.5×10^5 per well) were inoculated in a volume of 1.0 ml into 24-well plates (Corning Glass Works, Corning, N.Y.). Macrophages were allowed to adhere overnight. Bacteria were added in a small volume of L broth (10 to 50 µl) to obtain a multiplicity of infection of approximately 10:1. The tissue culture plates were centrifuged at 1,000 rpm for 5 min to synchronize infection and facilitate adhesion. After infection was allowed to proceed at 37°C for 30 min, the medium was removed from each well and 0.5 ml of 100 µg of gentamicin sulfate (Sigma, St. Louis, Mo.) per ml in DMEM-fetal bovine serum was added to each well. The plates were incubated at 37°C for 90 min. At the first time point (2 h total), the medium was removed and each well was washed with 0.5 ml of DMEM-fetal bovine serum twice. Then, 0.2 ml of 1% Triton X-100 (Calbiochem-Novabiochem Corp., La Jolla, Calif.) was added to each well for 5 min. After adding 0.8 ml of LB broth to each well, the lysates were collected. For later time points, the medium containing 100 µg of gentamicin per ml was replaced after 90 min with medium containing 10 µg of gentamicin per ml until the appropriate time before rinsing and lysis.

To quantitate the number of cell-associated bacteria, infected monolayers were washed with phosphate-buffered saline (PBS) three times after incubation for 5 or 10 min at 37°C. The monolayers were lysed in 0.5 ml of Triton X-100 for 5 min; lysates from triplicate or duplicate wells were collected, diluted, and plated after each well was rinsed with 0.5 ml of PBS.

Animal experiments. Six- to 8-week-old female BALB/c mice (Charles River Laboratory) or 16 to 20 lb (7.3 to 9.1 kg) DBA/1 or DBA/2 mice (Jackson Laboratory, Bar Harbor, Maine) were used for ileal loop infection experiments. Each mouse strain yielded equivalent results. To examine the interactions of salmonellae with murine intestinal epithelial cells, the ligated intestinal loop infection model was used. The procedures of Jones et al. were used (18). Briefly, the bacterial inoculum was delivered into the ligated ileal loop at a point that was anterior to the third Peyer's patch from the cecum. At various times postinfection, the mice were euthanized by cervical dislocation and the Peyer's patches were removed for processing.

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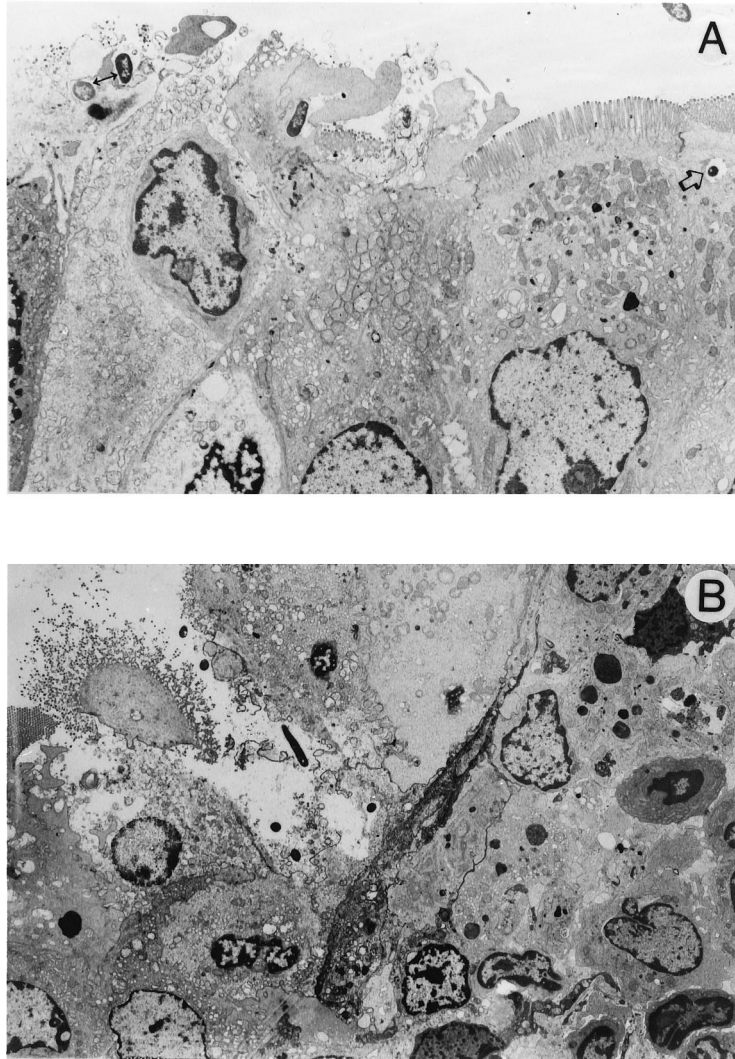
FIG. 1. Transmission electron micrographs of *S. typhimurium* infection of murine Peyer's patches at 30 min postinfection. (A) *S. typhimurium* entry into M cells is accompanied by dramatic cytoskeletal rearrangements. Multiple organisms entering a single M cell (double-headed arrow). Note the bacterium inside the vacuole of an M cell (open arrow). (B) *S. typhimurium* causes disruption of the Peyer's patch epithelium as it destroys M cells. Cellular debris is released into the lumen.

Preparation of samples for transmission electron microscopy (TEM). (i) Infected Peyer's patches. The procedure of Jones et al. was followed (18). Thick sections of fixed, infected Peyer's patch tissues were cut and stained to locate the domes of the Peyer's patches; thin sections were cut and examined with a Philips model 201c transmission electron microscope.

(ii) Infected tissue culture macrophages. RAW264.7 cells (10^5) were seeded onto round coverslips and allowed to adhere overnight. Cells were infected by using the procedure described above. At various times after infection, the cells were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer, (pH 7.2) for 30 min in the cold. Tissues were then postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 20 min, and stained with 1% aqueous uranyl acetate for 15 min. Samples were then extensively washed with water before dehydration through a graded series of ethanol washes. The samples were infiltrated with Poly/Bed 812 for 1 h. To embed the samples, gelatin capsules were filled with the resin and placed on top of the coverslip. The polymerization process was allowed to proceed for 16 h at 60°C before the removal of the glass coverslip. To complete the polymerization process, the blocks were incubated at 60°C for an additional 24 h. Then, serial sections were cut and examined with a Philips model 201c transmission electron microscope.

Preparation of samples for histology. For histological studies, the excised infected Peyer's patch tissues were fixed overnight in 10% formalin in PBS at room temperature. The samples were embedded in paraffin, sectioned, and stained with Giemsa (Fluka Chemie AG, Buchs, Germany) and May-Grunwald (Fluka Chemie AG) stains.

Time-lapse video microscopy. RAW264.7 cells (10^5) were seeded onto a coverslip (22 by 30 mm) and cultured overnight in DMEM-fetal bovine serum. The coverslip was assembled into a chamber (22 by 30 by 1 mm) and filled with medium containing 10 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Gibco BRL). The chamber was placed on an inverted microscope (Nikon Diaphot model 200) equipped with a temperature-controlled stage heated to 37°C. Images were collected with a video camera (Hamamatsu CCD model C2400 camera) mounted onto the microscope. Video images were taken at four frames per s with a Video8 recorder (Sony).

To follow the invasion process, an area of the coverslip containing three to five macrophages was selected and recorded for at least 1 min before the addition of the bacteria. To obtain a multiplicity of infection of 10:1, 10^6 bacteria in 10 μl of LB broth were added to one side of the chamber during recording. Image collection was continued over a period of at least 45 min after infection. In several experiments, the fate of intracellular bacteria was monitored for more than 2 h.

Photographs of still images from videos were captured by using an MRC 1000 system with COMOS software (Bio-Rad, Inc., Hercules, Calif.), and images were manipulated with Adobe (Seattle, Wash.) Photoshop software.

RESULTS

Behavior of various *Salmonella* strains within murine Peyer's patches. *Salmonella* interactions with the murine ileal Peyer's patch epithelium were observed by TEM of infected ligated ileal loops. Initially, *S. typhimurium* establishes infection by entering and destroying M cells (18), the specialized
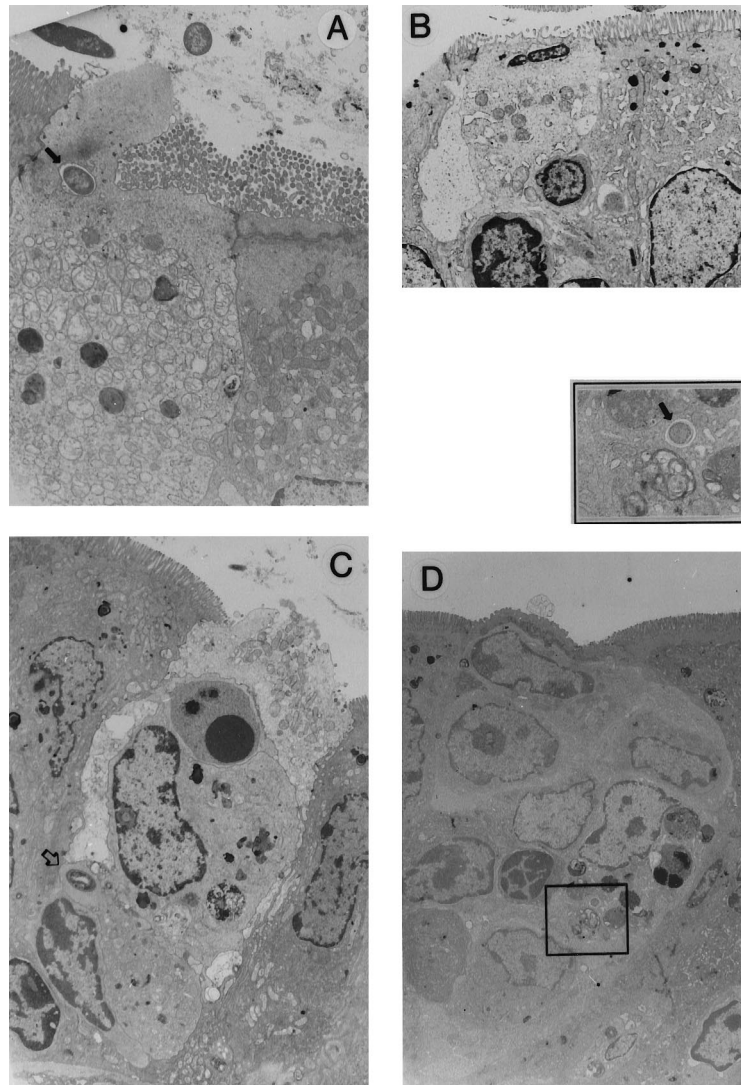
FIG. 2. Transmission electron micrographs of *S. typhi* infection of murine Peyer's patches at 60 min postinfection. (A) *S. typhi* entry into the M cell is accompanied by cytoskeletal rearrangement. The arrow points to the bacterium in a vacuole. (B) *S. typhi* is found within vacuoles of M cells. It appears that the microvilli of this *S. typhi*-invaded M cell are intact. (C) *S. typhi* (open arrow) is found within a vacuole of a phagocytic cell beneath the M cell. Note that the M cell is disrupted but has not extruded into the lumen. The epithelial layer is intact. (D) *S. typhi* is found within a vacuole of a cell beneath the M cell. Note that both the M cell and epithelial layer are intact. (Inset) Closeup view of *S. typhi* bacterium (boxed area) is shown above panel D (arrow).

microfold cells that together with enterocytes form the follicle-associated epithelium of the Peyer's patches. Entry of *S. typhimurium* into murine M cells is accompanied by major disruption of the M-cell cytoskeleton (Fig. 1A) (18). At 30 to 60 min postinfection, infected M cells are extruded into the bowel lumen, with a resulting loss of integrity of the Peyer's patch epithelium (Fig. 1B) (18).

Figure 2A shows *S. typhi* entering murine M cells. During both *S. typhi* and *S. typhimurium* infections, entry into murine M cells was accompanied by dramatic cytoskeletal rearrangement. After invasion, both species could be found in vacuoles (Fig. 2B). Although the numbers of bacteria were equal during each infection, fewer *S. typhi* organisms than *S. typhimurium* organisms were detected near or in M cells. Also, while many *S. typhimurium* organisms were found to interact with a single M cell, presumably because of passive entry (12), we observed only single *S. typhi* organisms interacting with any one M cell.

During *S. typhimurium* infection, extensive M-cell destruc-

tion resulted in the detachment of the Peyer's patch epithelial layer from the basal lamina. In contrast, there was no evidence of extensive M-cell damage or of epithelial layer destruction during *S. typhi* infections. Indeed, within 1 h, the majority of visible *S. typhi* organisms in the Peyer's patches were found within vacuoles of phagocytic cells beneath intact or relatively undamaged M cells (Fig. 2C and D). *S. typhimurium* invasion of murine Peyer's patches is accompanied by replication (18) while *S. typhi* invasion results in clearance from murine Peyer's patches (data not shown).

Histological sections of Peyer's patches infected with salmonellae for 2 and 3 h were examined by light microscopy. Because the entire follicle-associated epithelium could be visualized, we could better evaluate the extent of tissue damage beyond the epithelium. *S. typhimurium* caused generalized destruction of the murine follicle-associate epithelium, and the bacteria reached the muscularis mucosa. During *S. typhi* infection periods of 2 and 3 h there was no evidence of destruction
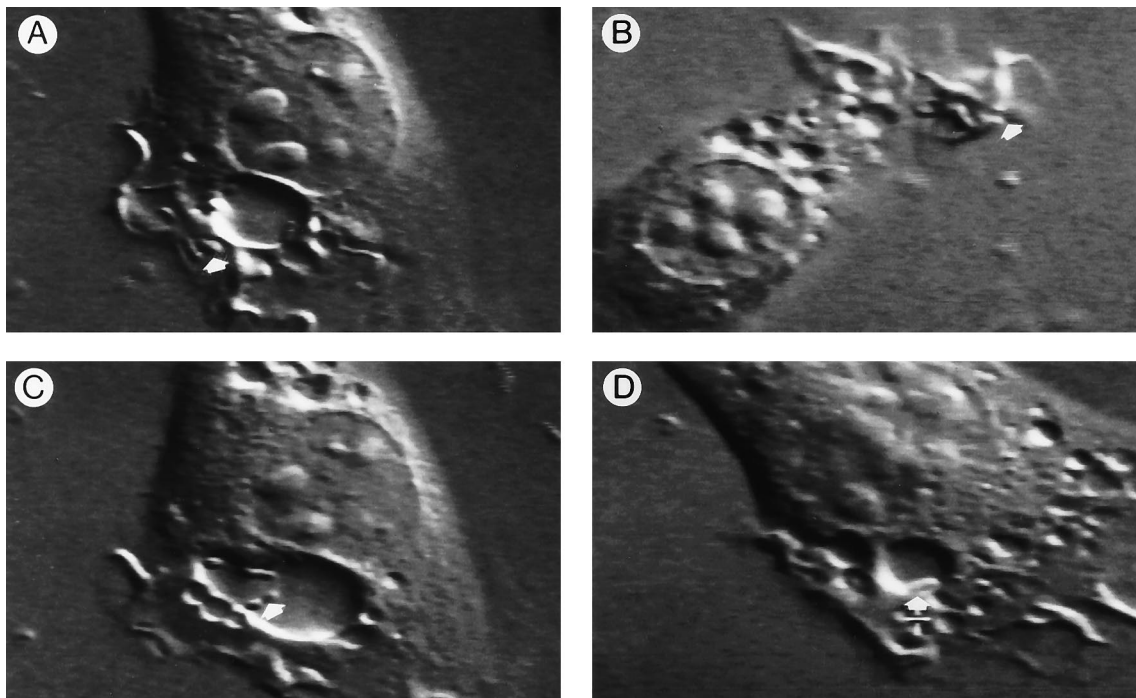
FIG. 3. Digitally captured images from video microscopy of *Salmonella*-infected RAW264.7 cells. (A) *S. typhimurium* entering RAW264.7 cells by macropinocytosis. Bacteria are indicated on either side of arrow. (B) Macropinocytosis by RAW264.7 cell in response to *S. typhi* infection. The bacterium is indicated by the arrow. (C) Fused macropinosome containing multiple organisms during *S. typhimurium* infection of RAW264.7 cells. Two organisms are indicated on either side of the arrow. (D) Macropinosome containing *S. typhi* (indicated by arrow) in an RAW264.7 cell.

nor were bacteria present beneath the follicle-associate epithelium (data not shown). *S. gallinarum* infection showed no evidence of bacterial entry or destruction of murine M cells, or of enterocytes, after 60 min of infection (data not shown).

Behavior in RAW264.7 cells observed by time-lapse video microscopy. The entry of salmonellae into RAW264.7 cells was observed by video microscopy. It has been previously shown that *S. typhimurium* induces macropinocytosis during entry and persists in spacious phagosomes within murine bone marrow-derived macrophages (1). Macropinosomes, or spacious vacuoles produced during macropinocytosis, are endocytic vesicles formed from plasma membrane ruffles in response to phorbol esters or growth factor stimulation (25, 26). While *S. typhimurium* entry into RAW264.7 macrophages was accompanied by dramatic ruffling of the macrophage membrane and macropinocytosis (Fig. 3A), *S. typhi* entry resulted in comparatively less dramatic ruffling and macropinocytosis (Fig. 3B). As was observed during M-cell entry, fewer *S. typhi* organisms than *S. typhimurium* organisms appeared to interact with a single cell. Overall, 50% of RAW264.7 cells observed became infected with *S. typhimurium*, whereas only 1 to 5% of the RAW264.7 cells became infected by *S. typhi* or *S. gallinarum* after a 2-h observation period.

Immediately following *S. typhimurium* entry, vacuoles containing bacteria fused with each other and with other macropinosomes to form spacious phagosomes (1) containing more than one bacterium (Fig. 3C). *S. typhi*-containing vacuoles were smaller in size and rarely showed fusion with other cellular vesicles (Fig. 3D).

There was no evidence of membrane ruffling or of macropinocytosis during entry of *S. gallinarum* or *E. coli* (data not shown). Also, extensive vacuole fusion was not noticeable during *S. gallinarum* or *E. coli* infection.

Comparisons of behaviors of *Salmonella* strains within macrophage-like cell lines by TEM. *Salmonella*-infected RAW 264.7 macrophages were analyzed by TEM at various times postinfection (30 min and 2, 5, and 22 h). Both *S. typhimurium* and *S. typhi* organisms were found in spacious vacuoles and in smaller vacuoles, where vacuole membranes were tightly apposed to the organisms within 30 min of infection (Fig. 4a and b). In contrast, *S. gallinarum* and *E. coli* were found exclusively in tightly apposed membrane bound vacuoles (Fig. 4c and d).

The spacious vacuoles containing *S. typhimurium* and *S. typhi* after 2 h of infection appeared to be smaller than the sizes after 30 min of infection (data not shown).

All bacteria were found in individual vacuoles with tightly apposed membranes after 22 h of infection of RAW264.7 cells. Whereas numerous *S. typhimurium* (Fig. 5a) and *S. typhi* (Fig. 5b) organisms could be detected intracellularly within individual macrophages, generally only one or a few intracellular *S. gallinarum* or *E. coli* organisms were found (Fig. 5c and d).

Comparative entry and growth of *Salmonella* species in a macrophage-like cell line. RAW264.7 cells were infected with each *Salmonella* species for 5 to 10 min, extracellular bacteria were removed by washing with PBS, and the infected monolayers were lysed and plated onto LB agar. Three experiments yielded similar results: 2 to 7% of the inoculum of *S. typhimurium* was cell associated whereas 0.2 to 0.7% of the inocula of *S. typhi* and *S. gallinarum* were cell associated (Table 1). These data suggest that *S. typhimurium* adheres more efficiently to and/or enters more rapidly into RAW264.7 cells than *S. typhi* or *S. gallinarum*.

The results of gentamicin protection assays revealed that both *S. typhimurium* and *S. typhi* underwent approximately 3.5 doublings between 2 and 24 h of infection whereas the num-
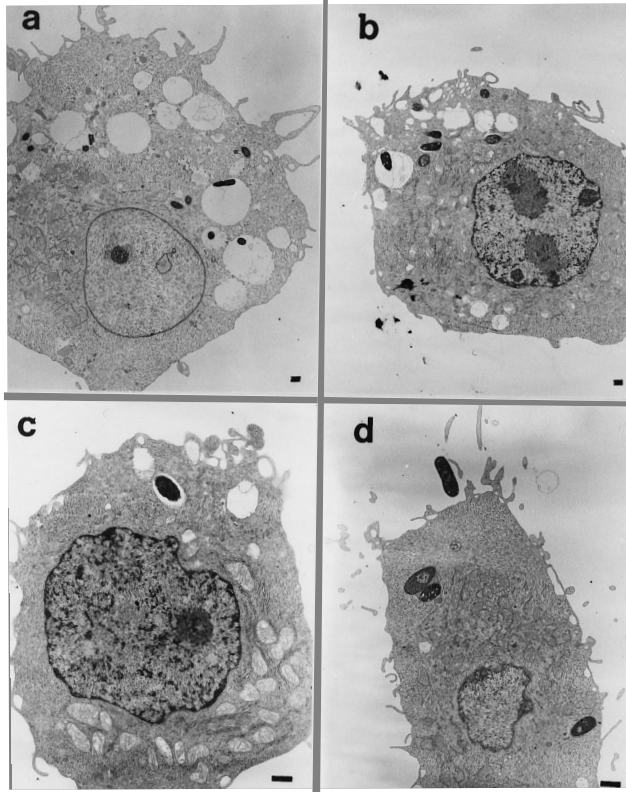
FIG. 4. Transmission electron micrographs of *Salmonella*-infected RAW 264.7 cells at 30 min postinfection. Individual *S. typhimurium* (a) and *S. typhi* (b) organisms are present in spacious vacuoles and in vacuoles with tightly apposed membranes. In panel a, a dividing bacterium is visible in a spacious vacuole. (c) Individual *S. gallinarum* organisms are present in smaller vacuoles and in vacuoles with tightly apposed membranes. (d) An *E. coli* bacterium is present in a vacuole with tightly apposed membranes.

bers of intracellular *S. gallinarum* and *E. coli* organisms decreased during the time course (Fig. 6).

These combined data suggest that although *S. typhi* associates with RAW264.7 cells less efficiently than *S. typhimurium*, *S. typhi* is capable of persisting within RAW264.7 cells. In contrast, *S. gallinarum* is incapable of efficient entry into and persistence within RAW264.7 cells.

DISCUSSION

In this study we have attempted to identify the barriers to infection in the mouse for *S. typhi* and *S. gallinarum*, using both in vitro and in vivo experiments. Earlier work in the field revealed that *S. typhi* does not infect the mouse by any route of inoculation (6) whereas *S. gallinarum* may transiently infect the mouse when inoculated intravenously or intraperitoneally but not when inoculated orally (2, 6). On the basis of the findings that Peyer's patches of human typhoid fever patients are ulcerated, we assume that *S. typhi* enters and destroys the human Peyer's patch epithelium in the same manner as does *S. typhimurium*: by active entry into the M cells (17, 24). It is not known whether ileal lymphoid tissue is the site of initial interaction during *S. gallinarum* infection in chickens. The fact that *S. typhi* enters murine M cells and *S. gallinarum* does not confirms that *Salmonella* host restriction is complex and diverse.

The work described here reveals that *S. typhi* enters and may

be found within vacuoles of murine M cells and confirms the earlier observations that *S. typhi* causes cytoskeletal rearrangements in murine M cells (19). In comparison to *S. typhimurium*, *S. typhi* enters M cells less efficiently and never disrupts the integrity of the bowel epithelial barrier. The facts that most of the observed *S. typhi*-containing M cells are intact and few, if any, *S. typhi* organisms are detected in the Peyer's patches soon after infection show a clear distinction between *S. typhi* and *S. typhimurium*. One reason why *S. typhi* infections are cleared from murine Peyer's patches while *S. typhimurium* infections are not may be because differential macrophage activation results from entry of the two species. For example, since *S. typhi*-invaded M cells are not destroyed, these M cells may secrete cytokines that activate the underlying macrophages to respond effectively to *S. typhi* infection. We plan to test this hypothesis by measuring and comparing cytokine secretion in *Salmonella*-infected murine Peyer's patches.

At first glance, these data appear to be inconsistent with the findings of Kohbata et al. (19), who showed that *S. typhi* destroys murine M cells during 30-min ileal loop infections. We believe that the inconsistency lies in differences in experimental design and interpretation. On rare occasions, we found that 30-min *S. typhi* infections were associated with murine M-cell destruction. But, during infection periods longer than 30 min, we observed *S. typhi* within cells that were underneath relatively healthy M cells. It is possible that the 10-fold-higher inocula of *S. typhi* organisms used by the Kohbata group resulted in comparatively greater M-cell destruction. Alterna-
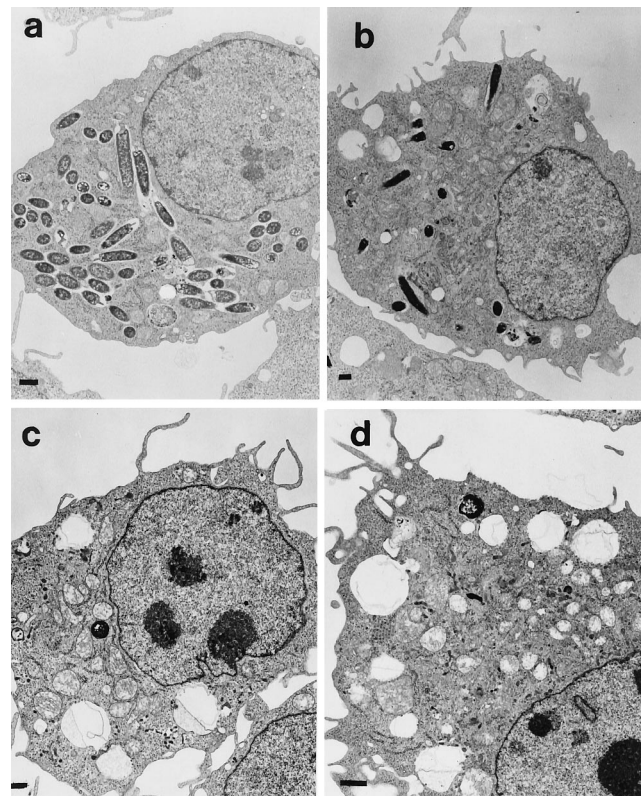

FIG. 5. Transmission electron micrographs of *Salmonella*-infected RAW 264.7 cells 22 h postinfection. Individual *S. typhimurium* (a) and *S. typhi* (b) organisms are present in vacuoles with tightly apposed membranes. In panel a, some bacteria appear disrupted. A single *S. gallinarum* (c) or *E. coli* (d) organism is present in a vacuole with tightly apposed membranes. The bacterium appears disrupted.

TABLE 1. Comparisons of macrophage adhesion and entry of *Salmonella* species in RAW264.7 cells[a]

Species	% of cell-associated organisms		
	Expt 1 (5 min)	Expt 2 (5 min)	Expt 3 (10 min)
S. typhimurium	7.1	2.4	3.0
S. typhi	0.7	0.2	0.5
S. gallinarum	0.6	0.5	0.3

[a] Monolayers of RAW264.7 cells in 24-well tissue culture plates were infected with each *Salmonella* species in triplicate for the indicated times. Dilutions of each lysate were plated onto LB agar, and the mean number of CFU from triplicate or duplicate wells was calculated (lysate). Standard deviations did not exceed 26% of the means, except in one case where the standard deviation of the mean for the *S. typhi*-infected lysate in experiment 3 was 36%. The percentage of cell-associated organisms was calculated as lysate/inoculum × 100.

tively, the cytoskeletal rearrangements, perceived as damage by the Kohbata group, may heal after *S. typhi* enters and/or translocates across the M cell. Our data confirm Kohbata et al.'s observations that *S. typhi* infection of murine Peyer's patches does not result in the destruction of enterocytes and therefore does not cause extensive epithelial layer damage.

When comparing entry of *S. typhimurium* and *S. typhi* into M cells in vivo and macrophage cell lines in vitro, the common theme is that entry of *S. typhi* elicits a less pronounced cytoskeletal rearrangement and fewer *S. typhi* organisms enter. Entry into RAW264.7 cells, however, is characterized by macropinocytosis for both *S. typhimurium* and *S. typhi*. Within RAW264.7 cells, *S. typhimurium* and *S. typhi* grow at the same rate, but the total number of *S. typhimurium* organisms is greater than the total number of *S. typhi* organisms after 24 h of infection because the uptake of *S. typhimurium* is more

efficient. If experiments with cultured cells accurately model the in vivo infection, it is possible that *S. typhi* is cleared more rapidly simply because fewer organisms are taken up. It is possible, however, that macrophages are not responsible for rapid clearance of salmonellae from Peyer's patches or that experiments with cultured macrophages do not accurately reflect the state of macrophages in the Peyer's patches.

At least one barrier to the establishment of *S. gallinarum* infection in the mouse appears to be the bacterium's inability to enter the murine intestinal epithelium. It is possible, however, that *S. gallinarum* may invade M cells or enterocytes at frequencies too low for us to detect in our experiments. Observations that a small percentage (approximately 0.001%) of an oral dose of *S. gallinarum* enters the murine liver after 4 days of infection support this possibility (2). The fact that *S. gallinarum* does not persist within RAW264.7 cells is consistent with the finding that *S. gallinarum* does not persist within the murine liver or spleen after intravenous infection (2). Although the site of replication of salmonellae within the liver is controversial, it is likely that the macrophage plays a role in infection (7, 21, 23).

Each *Salmonella* species tested was grown under conditions that are optimal for *S. typhimurium* invasion of cultured epithelial cells (20). It is possible that the optimal growth conditions differ among *S. typhimurium*, *S. gallinarum*, and *S. typhi* for the tested in vitro phenotypes. Reports of the optimal growth conditions for *S. typhi* to invade cultured epithelial cells are conflicting (22, 27) and, in one case, are shown to be the same for *S. typhimurium* and *S. typhi* (22). *S. gallinarum* naturally infects avian hosts, whose body temperatures are 5°C higher than those of mammals. It is likely that the different environmental cues encountered by *S. typhimurium*, *S. gallinarum*, and *S. typhi* during natural infection play a role in host specificity.

The phenotypes that we have described for entry and persistence of *S. typhi*, *S. gallinarum*, and *S. typhimurium* in cultured murine macrophages and in murine Peyer's patches suggest both in vitro and in vivo experimental approaches to identifying the genetic and molecular bases of host specificity. This work is in progress.
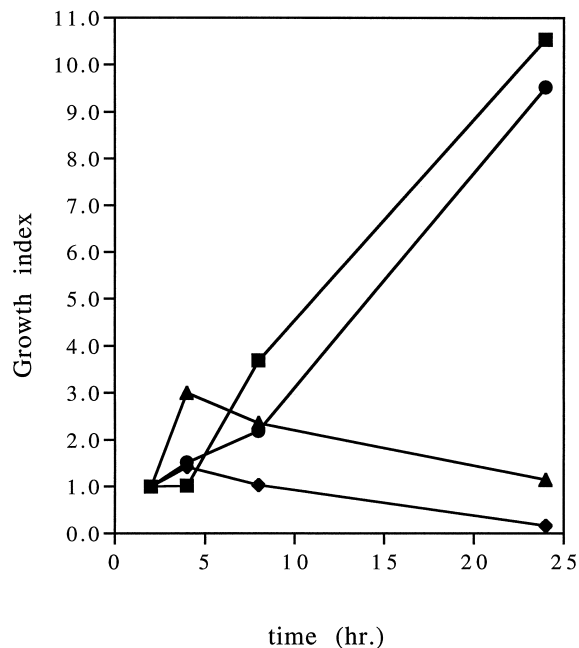
FIG. 6. Growth and persistence of *Salmonella* species in RAW264.7 cells. The percentage of intracellular bacteria was calculated as the number of gentamicin-resistant CFU per inoculum × 100. The growth index was defined as the ratio of the percentage of intracellular bacteria at 2 h postinfection to the percentage of intracellular bacteria at the indicated time. ■, *S. typhimurium*; ●, *S. typhi*; ▲, *S. gallinarum*; ◆, *E. coli*.

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