INFECTION AND IMMUNITY, July 1996, p. 2474–2482 Vol. 64, No. 7
0019-9567/96/$04.00+0

Analysis of Epithelial Cell Stress Response during Infection by *Shigella flexneri*

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Received 20 December 1995/Returned for modification 7 March 1996/Accepted 11 April 1996

***Shigella flexneri*-infected macrophage cells undergo an apoptotic-like death as early as one hour after infection (A. Zychlinsky, M. C. Prévost, and P. J. Sansonetti, Nature [London] 358:167–168, 1992). To determine the fate of infected epithelial cells, we characterized the viability, morphology, and several metabolic activities of HeLa cells after treatment with M90T, an invasive isolate of *S. flexneri* serotype 5, or BS176, a noninvasive isolate cured of the 220-kb virulence plasmid. Using standard assays, we found that for at least 4 h after infection with M90T, HeLa cells remained viable and did not detach or lyse. The ultrastructural morphology of HeLa cells heavily infected with M90T was free of hallmarks associated with cells undergoing apoptosis. Consistent with the idea that intracellular bacterial growth is metabolically stressful to the host cell, we observed that, compared with BS176 treated-HeLa cells, M90T-treated HeLa cells showed (i) a significant decrease in the total pool size of nucleoside triphosphates, (ii) a reduced ability to incorporate extracellular radiolabeled methionine into the soluble and insoluble cell fractions, and (iii) a stimulation of glucose uptake. However, there was no detectable increase in expression of the stress-inducible *hsp70* gene in M90T-infected HeLa cells or activation of the anaerobic metabolic pathway as determined by measuring total lactate levels. These results demonstrate clearly that the fate of *S. flexneri*-infected cells can vary dramatically between cell types and agree with the hypothesis that the destruction of epithelial cells observed in experimental models of shigellosis is due to the host inflammatory response and probably not bacterial intracellular multiplication per se.**

Shigellosis, or bacillary dysentry, remains a worldwide health threat responsible for the death of more than 500,000 children each year in developing countries (2). Clinical symptoms of shigellosis range in severity but most typically resemble an intense acute inflammatory response with blood, mucus, and polymorphonuclear cells in the stool and often severe degeneration of the colonic epithelium (11). The gram-negative bacterium *Shigella flexneri* is the etiological agent of the most common endemic form of bacillary dysentery. Pathogenesis involves the penetration of the bacterium into the epithelial cells of the colon (20), multiplication therein, and then spread to adjacent cells (11). The molecular and cellular basis of pathogenesis has been studied by using limited laboratory animal models and in vitro-grown mammalian cell lines which are susceptible to infection (13). Certain aspects of pathogenesis, including epithelial cell invasion and intracellular spread, have been reviewed recently (9, 31).

Entry of *S. flexneri* into epithelial cells is mediated by at least three proteins, IpaB, IpaC, and IpaD, which are encoded on a large 220-kb virulence plasmid (16, 25, 26, 34, 40). Upon contact with epithelial cells grown in culture, *S. flexneri* induces massive host cell cytoskeletal rearrangements, termed ruffles, which result in bacterial engulfment (1, 6), and activates the host cell signalling pathway(s) (8). Within minutes after being internalized, *S. flexneri* lyses the phagosomal vacuole and is released into the host cell cytoplasm (33). *S. flexneri* can multiply within the cytoplasm of HeLa cells with an estimated doubling time of 40 min (33). A subpopulation of intracellular bacteria express on their cell surface the plasmid-encoded IcsA (VirG) protein, which allows both spread of the bacteria within the cytoplasm and dissemination into adjacent cells via finger-like protrusions (4, 21).

In addition to colonocytes, *S. flexneri* can infect other cell types present within the mucosa, including macrophages. The effect of *S. flexneri* infection on J774 murine macrophage cells has been studied in detail. Within an hour after infection, host cell metabolism is dramatically altered and the cells detach or lyse (32). The ultrastructural morphology of infected macrophages includes condensation of chromatin at the nuclear boundry, blebbing at the cell surface, dilation of the endoplasmic reticulum, and cytoplasmic vacuolization and is identical to the morphology of cells undergoing apoptosis, or programmed cell death (45). This apoptotic killing of macrophages is not a simple consequence of bacterial multiplication within the cytoplasm but is specific to invasive *S. flexneri*, which expresses the virulence plasmid-encoded IpaB protein (44). *S. flexneri* infection also stimulates macrophage cells to release interleukin-1β, which may function in vivo in initiating or potentiating the host inflammatory response (43).

It is proposed that *Shigella* multiplication within the cytosol of colonic epithelial cells causes host cell destruction, lysis, or detachment from the basement membrane matrix (11). However, there is emerging evidence from animal models of shigellosis that the host immune response is the primary cause of tissue destruction (29). To determine the fate of infected epithelial cells, we characterized the viability, morphology, and several metabolic activities of HeLa cells after treatment with M90T, an invasive isolate of *S. flexneri* serotype 5, or BS176, a noninvasive isolate cured of the 220-kb virulence plasmid. Using standard cytotoxicity assays, we found that HeLa cells infected with *S. flexneri* M90T remained viable and did not detach or lyse for as long as 4 h postinfection. Furthermore, these cells were free of the ultrastructural hallmarks associated with cells undergoing apoptosis. However, *S. flexneri*-infected cells showed biochemical changes characteristic of cells undergoing

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metabolic stress. These results indicate that the fate of *S. flexneri*-infected cells can be dramatically different depending on the host cell type and agree with the emerging hypothesis that the destruction of epithelial cells observed in experimental models of shigellosis is due to the host inflammatory response and probably not intracellular multiplication per se.

MATERIALS AND METHODS

Bacterial strains and cell lines. Strain M90T is an invasive *S. flexneri* serotype 5 isolate which contains the virulence plasmid pWR100 (33). Strain BS176 is a plasmid-cured derivative of M90T. Bacteria were routinely cultured in Trypticase soy broth (Diagnostics Pasteur, Marnes-La-Coquette, France) in a shaking incubator at 37°C or on Trypticase soy broth-Bacto Agar (1.5%; Difco Co., Detroit, Mich.) plates containing Congo red (0.01% [wt/vol]) as described previously (23). The human uterine carcinoma cell line HeLa (ATCC CCL 2) was grown at 37°C in 5% CO_2 in minimal essential medium with L-glutamine (2 mM) (MEM; Gibco BRL, Gaithersburg, Md.) supplemented with 10% fetal calf serum (Gibco BRL).

Infection of HeLa cells. The protocol is described in detail elsewhere (33). Briefly, semiconfluent HeLa cells, grown in either 35-mm-diameter petri dishes or 12-well microtiter plates, were overlaid with a mid-log-phase culture of *S. flexneri* suspended in MEM at a concentration of 10^5 CFU/ml, centrifuged for 10 min at 2,200 × *g* to promote adherence of bacteria to HeLa cells, and then incubated for 45 min at 37°C in 5% CO_2. Cells were then washed four times with prewarmed Earle's balanced salt solution, overlaid with MEM containing gentamicin (50 μg/ml), an antibiotic which efficiently kills extracellular but not intracellar bacteria, and returned to the incubator. The addition of gentimicin was defined as time zero for all experiments. HeLa cells were washed four times with prewarmed phosphate-buffered saline (PBS) prior to any further manipulations. The efficiency of infection was determined by visually scoring ethanol-fixed, Giemsa-stained cells (Fig. 1) (33).

Microscopic analysis of infected cells. For light microscopy analysis, HeLa cells were fixed for 10 min in paraformaldehyde (3.7% in PBS [pH 7.4]) and then stained with Giemsa stain (diluted 1:20 in water). HeLa cells grown in 35-mm-diameter petri dishes were fixed and prepared for electron microscopy, using the standard protocols described previously (33). Mitochondria were visualized by immunofluorescent labeling and confocal scanning microscopy, using a standard protocol described previously (8); polyclonal rabbit antiserum to the yeast mitochondrial ADP/ATP carrier protein was kindly provided by the laboratory of Walter Neupert (University of Munich, Munich, Germany).

HeLa cell cytotoxicity assays. At indicated times after infection, HeLa cells were washed four times with PBS, overlaid with trypan blue (0.2% [wt/vol] in PBS) for 1 min, and then scored visually, using an inverted microscope. A minimum of 500 cells per plate were counted, and each experiment was done in duplicate.

To detect HeLa cell detachment or lysis, HeLa cells were grown for 18 h in the presence of [5-^{3}H]uridine (0.5 μCi/ml; >26 Ci/mmol; Amersham Life Sciences, Buckinghamshire, England), washed with MEM, and then infected as described above. At indicated times after infection, total culture supernatant was collected and diluted 1:10 into BCS scintillation fluid (Amersham Life Sciences); a fraction was then assayed for counts per minute in a Beckman scintillation counter. To determine the amount of radioactivity remaining on the culture plate, cells were washed four times with PBS, overlaid with 1 ml of lysis buffer (0.5% deoxycholate [wt/vol] in water), collected with a cell scraper, and diluted into BCS scintillation fluid as described above. Lactate dehydrogenase activity present in the culture supernatant of HeLa cells was measured exactly as described previously for macrophage cells (43).

Estimation of NTP levels. At indicated times after infection, HeLa cells were washed with PBS, treated with trypsin for 5 min at 37°C, collected with a Pipetteman, carefully transferred to an Eppendorf tube, and then collected by gentle centrifugation (800 × *g*) at 4°C. The resulting pellet was resuspended in 0.125 ml of trichloroacetic acid (TCA; 8%) followed by an equal volume of water, incubated on ice for 5 min, and then centrifuged at 14,000 × *g*. The cleared supernatant was used immediately as a source of substrate in a quantitative colorimetric ATP assay performed as recommended by the manufacturer (Sigma Chemical Company, St. Louis, Mo.) and as done previously in our laboratory (32). The results are presented as relative units, where 1 U corresponds to approximately 1 μmol of nucleoside triphosphate (NTP).

Measurement of methionine incorporation into infected HeLa cells. We used a modification of the protocol of Hale and Formal (12). HeLa cells (10^5/ml) were grown in 12-well microtiter dishes and infected as described above. Twenty minutes prior to each time point indicated, HeLa cells were washed twice with Earle's balanced salt solution (37°C) and then overlaid with MEM lacking methionine (ICN Biochemicals, Irvine, Calif.) supplemented with 1 μCi of an [L-^{35}S]methionine mixture per ml (final concentration, 1 μM methionine; >1,000 Ci/mmol; Tran^{35}S-label; ICN Pharmaceuticals). Cells were then returned to the incubator for 20 min, washed four times with PBS, lysed with 0.5 ml of deoxycholate (0.5% [wt/vol] in H_2O), collected with a cell scraper, and transferred to an Eppendorf tube. A fraction (0.1 ml) was removed, and the number of counts per minute was determined in a Beckman scintillation counter. This activity was defined as total activity. The remaining 0.4 ml was centrifuged (14,000 rpm) for 5 min, and a fraction of the supernatant was removed to a new Eppendorf tube containing TCA to a final concentration of 10%. The mixture was allowed to precipitate overnight at −20°C, centrifuged, vacuum dried, then solubilized in BCS solution, and assayed for counts per minute. The results are presented as (cpm from M90T treated cells/cpm from BS176 treated cells) × 100. The incorporation of radioactivity into BS176-treated cells was identical to the incorporation observed in cells treated with medium alone (data not shown).

Uptake of glucose into infected HeLa cells. HeLa cells were prepared in 12-well microtiter dishes and infected as described above for methionine incorporation assays. Twenty minutes prior to indicated times after infection, cells were removed from the incubator, washed twice with PBS (37°C), overlaid with glucose-free MEM (ICN Pharmaceuticals) supplemented with 1 μCi of D-[6-^{3}H]glucose (ICN Pharmaceuticals), and then returned to the incubator. Cells were washed four times with PBS to remove unincorporated glucose, then lysed with deoxycholate (0.5% [wt/vol] in water), collected with a cell scraper, and assayed for total counts per minute in a scintillation counter. All experiments were done in triplicate, and the results are the averages of three independent experiments.

Measurement of lactate levels in HeLa cells. Semiconfluent monolayers of HeLa cells grown in 60-mm petri dishes were infected as described above. At indicated times after infection, cells were washed with ice-cold PBS, hypoosmotically lysed by addition of 1.2 ml of water, and collected with a cell scraper. The cells were transferred to an Eppendorf tube and centrifuged for 6 min at 14,000 rpm at 4°C. A fraction of the supernatant (0.8 ml) was added to Sigma Lactate Reagent (Sigma Diagnostics, St. Louis, Mo.), and lactate levels were determined as described by the manufacturer. All experiments were done in triplicate, and each time point represents at least two independent experiments.

RNA isolation and analysis of *hsp70* mRNA levels by PCR. We used a modification of the protocol described by Sewing et al. (36) to measure *hsp70* mRNA levels. HeLa cells (2×10^5) were infected as described above and at times indicated were washed four times with ice-cold PBS; total RNA was isolated by using RNAsol B solution exactly as described by the manufacturer (Bioprobes Systems, Montreuil-Sous-Bois, France). RNA (ca. 6 μg/ml) was treated with RNase-free DNase (Promega, Madison, Wis.) for 10 min at 4°C and then heated for 5 min at 70°C to stop the reaction. For cDNA synthesis, RNA was denatured by incubation for 10 min at 60°C and then reverse transcribed in a solution containing First Strand buffer (Gibco BRL), 2,000 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL) per ml, 1,000 U of rRNasin RNase inhibitor (Promega) per ml, 1 mM each dNTP (Pharmacia, Uppsala, Sweden), and 20 μg of random hexamer primers (Boehringer, Mannheim, Germany) per ml in a final volume of 100 μl at 42°C for 1 h. Ten microliters of this reaction was used as the template in a standard PCR performed with 0.2 μCi of alpha-labeled dCTP per ml and oligonucleotide primers against the *hsp70* (18) or *grp78* (37) gene. The 5′ primer (-ATCGAGGTGACCTTCGACATC-) and the 3′ primer (-ACACACCTGCTCCAGCTCCTT-) against *hsp70* were synthesized by the Unité Chimique Organic, Institut Pasteur. PCR products were size fractionated on a standard denaturing 6% polyacrylamide DNA sequencing gel and exposed on Kodak X-Omat audoradiographic film at −80°C.

RESULTS

Kinetics of *S. flexneri* infection of HeLa cells. Before an analysis of the HeLa cell response to *S. flexneri* infection was begun, it was necessary to establish a standardized and reproducible infection assay. Optimized conditions for the infection of HeLa cells by *S. flexneri* have been previously described (12, 33), and we used a protocol in which a semiconfluent monolayer of HeLa cells was overlaid with a suspension of bacteria (multiplicity of infection, 150), gently centrifuged to promote bacterium-cell adhesion, incubated for 45 min, and then washed and overlaid with medium containing the antibiotic gentimicin to stop further infection. We defined the addition of gentimicin as time zero. We monitored the kinetics of infection two ways. First, at 1-h time points, we determined the percentage of total HeLa cells which were infected by visually inspecting fixed Giemsa-stained cells. Second, because the antibiotic gentimicin efficiently kills extracellular bacteria but not bacteria present within the host cell cytoplasm, the number of CFU obtained from gentle lysis of the HeLa cell monolayer at different times postinfection can be used to estimate the total number of intracellular bacteria and intracellular bacterial growth rates (12). A representative experiment is shown in Fig. 1.

By 1 h after infection with invasive *S. flexneri* M90T, about 40 to 50% of all HeLa cells contained one or more bacteria (Fig.

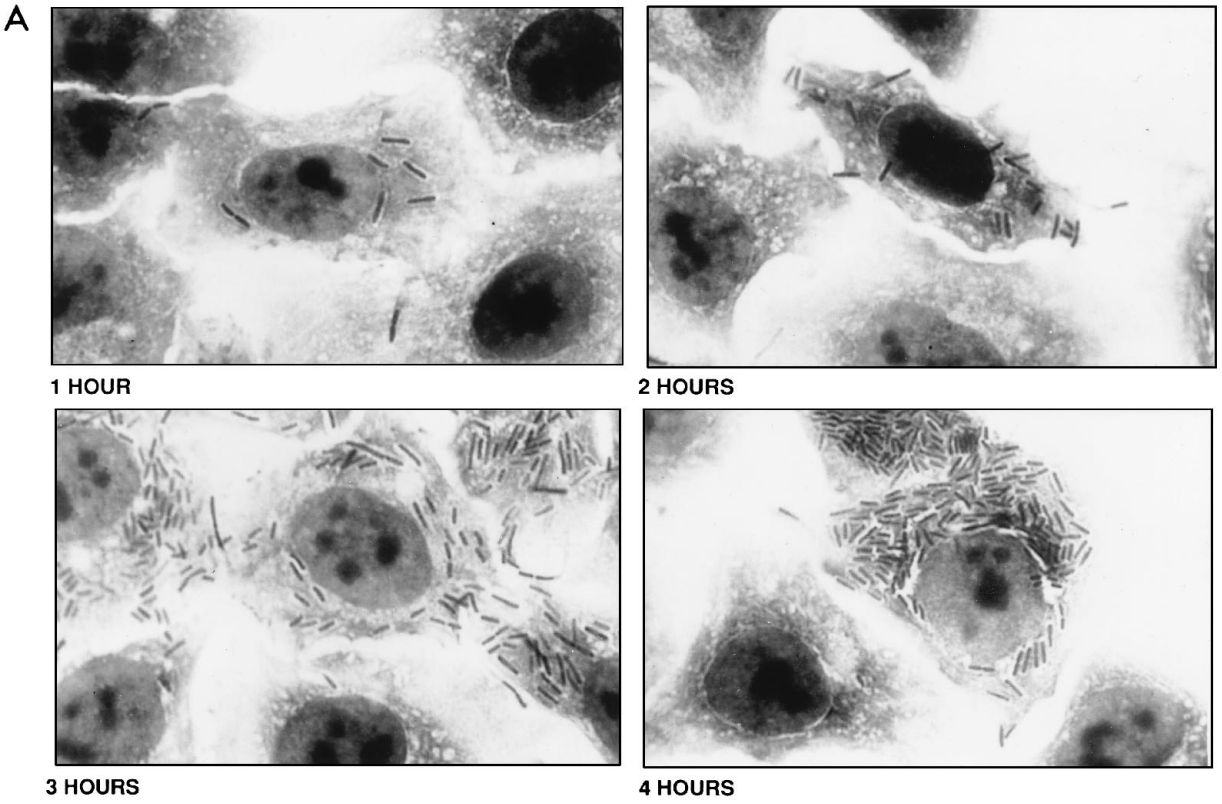

FIG. 1. Infection of HeLa cells by *S. flexneri* M90T. HeLa cells were infected with invasive strain M90T as described in Materials and Methods and at indicated times postinfection were fixed in paraformaldehyde (3.7% in PBS) and then stained with Giemsa. Magnification, ×100. (B) At various times postinfection (i.e., after addition of gentimicin), HeLa cells were lysed with deoxycholate and serial dilutions were plated on nonselective Trypticase soy broth-agar. Plotted is the number of CFU per 10^5 HeLa cells versus time. The results are the averages of one representative experiment done in triplicate.

1A). These bacteria were probably free within the cytoplasm, since lysis of the phagocytic vacuole occurs within minutes after engulfment (33). By 2 h postinfection, the number of cells which contained intracellular bacteria increased to between 60 and 80%, reflecting the ability of *S. flexneri* to spread from cell to cell via membrane-bound protrusions (4, 21). The number of intracellular bacteria increased for the duration of the 4-h experiment (Fig. 1B), and by 4 h, we observed HeLa cells completely packed with bacteria (Fig. 1A). When the same experimental protocol was performed with *S. flexneri* BS176, a noninvasive derivative of M90T that is cured of the virulence plasmid, less than 1% of the total number of HeLa cells contained bacteria after as long as 4 h postinfection (data not shown). Using this standardized protocol, we then investigated the viability, ultrastructural morphology, and stress response of HeLa cells after treatment with either BS176 or M90T.

Viability of *S. flexneri*-infected HeLa cells. Rapidly after infection with *S. flexneri*, macrophage cells lose the ability to exclude the dye trypan blue and then undergo lysis (32). To test whether HeLa cells remain viable after *S. flexneri* infection, we scored HeLa cells for the ability to exclude trypan blue each hour for 4 h after treatment with the noninvasive strain, BS176, or the invasive strain, M90T. We found that more than 95% ± 2% of untreated HeLa cells were able to exclude trypan blue. After treatment with BS176, this percent did not change. Surprisingly, we also found that after treatment with M90T, the proportion of HeLa cells which remained able to exclude the dye trypan blue remained greater than or equal to 95% ± 3%. This result suggests that the cells remaining on the culture plate after infection with M90T remained viable.

To test whether *S. flexneri* induced HeLa cell lysis or detachment, we metabolically labeled HeLa cells with [5-^{3}H]uridine for 18 h as described in Materials and Methods and then treated them cells with M90T or BS176. This treatment had no effect on the ability of M90T to infect these cells (data not shown). The amount of radioactivity remaining on the culture plate (Fig. 2) or the activity released into the supernatant (data not shown) was measured every hour for 4 h after infection. There was no detectable loss of radioactivity from the culture plate of HeLa cells treated M90T compared with control cells (medium alone) or cells treated with the noninvasive strain BS176 (Fig. 2). In agreement with this observation, the amount of radioactivity present in the cell culture supernatant of in-

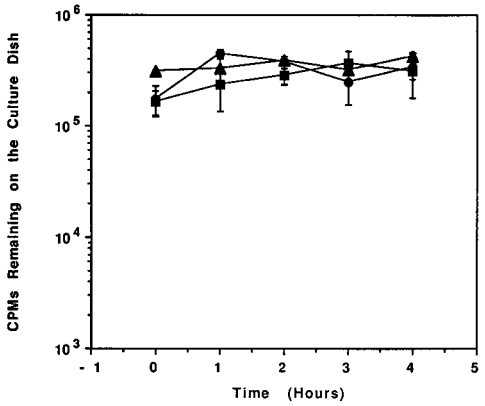

FIG. 2. HeLa cell detachment and lysis following infection with *S. flexneri*. HeLa cells were cultured for 18 h in the presence of [5-^{3}H]uridine and then treated with the noninvasive *S. flexneri* strain, BS176, or the invasive strain, M90T, as described in Materials and Methods. At indicated times postinfection, we determined the total amount of radioactivity present in the supernatant (data not shown) or the total amount of radioactivity remaining on the culture dish. Treatments: none (solid square); BS176 (solid circle); M90T (solid triangle). The results are the averages of four independent experiments.

fected cells was identical to that of control cells (data not shown). To verify these results, the culture supernatants of infected or noninfected HeLa cells were assayed for the presence of the cytoplasmic enzyme lactate dehydrogenase, using a protocol previously developed in the laboratory (43). There was no detectable difference in the amount of lactate dehydrogenase present in the supernatants of M90T treated cells compared with noninfected or BS176-treated cells. Together, these data indicate that HeLa cells, in sharp contrast to macrophage cells, remain viable and intact for at least 4 h after infection with *S. flexneri.*

Ultrastructure of infected HeLa cells. *S. flexneri*-infected J774 murine macrophages undergo ultrastructural morphological changes characteristic of cells undergoing apoptosis (45). To determine whether infected HeLa cells show any characteristics of programmed cell death, we analyzed by electron microscopy HeLa cells which had been infected with M90T for 3 h (Fig. 3a and b). As in previous studies (12, 33), we observed HeLa cells which contained a large number of intracellular bacteria, which are clearly visible by this technique and easily distinguishable from host cell organelles. The morphology of heavily infected cells was similar to that of uninfected cells with the exception of the presence of numerous electron-translucent vacuoles. These vacuoles have been noted previously (13). Infected cells were free of ultrastructural hallmarks characteristic of cells undergoing apoptosis (19). In particular, the chromatin was well dispersed and showed no evidence of compaction or segregation, the cytoplasmic membranes were intact and free of significant membrane blebbing, and the host cell organelles appeared normal. Results for HeLa cells analyzed at different times postinfection (i.e., 1, 2, or 4 h) were identical (data not shown).

It is interesting that throughout the course of this electron microscopy study, we consistently observed *S. flexneri* in close proximity to mitochondria (Fig. 3d), and in some cases there appeared to be membrane-membrane contact. This close association between *S. flexneri* and mitochondria has been observed previously in infected macrophage cells (7). We further investigated this phenomenon by double-immunofluorescence confocal scanning microscopy, in which we labeled both intracellular bacteria and mitochondria. Consistent with the transmission electron microscopy study described above, we did not detect any gross alterations in mitochondrial organization during bacterial invasion, although we often observed bacteria in close proximity to mitochondria (data not shown).

Is *S. flexneri* infection stressful to HeLa cells? The results above suggest that *S. flexneri* can multiply to high numbers within the cytoplasm of HeLa cells without inducing major host cell damage, detachment, or lysis. However, we hypothesized that the presence of intracellular, metabolically active bacteria may disrupt cellular homeostasis and metabolism. The response of HeLa cells to environmental stress is well studied (28, 42), and the following characteristics are observed in cells which are metabolically impaired: (i) reduced levels of ATP, (ii) inhibition of protein synthesis, (iii) stimulation of hexose transport, (iv) increase in the anaerobic respiratory pathway, and (v) transcriptional induction of the members of the *hsp70* gene family, including *hsp70* and *grp78*. We tested whether HeLa cells showed any of these characteristics after treatment with *S. flexneri.*

NTP levels decrease in infected HeLa cells. The intracellular pool of NTPs is often used as a measure of general cellular metabolic state, and the pool size is known to decrease after treatments that are metabolically stressful to the cell (42). We measured the total NTP level in infected HeLa cells at time zero (see Materials and Methods) and at 1, 2, and 3 h after infection with M90T and compared it with the level observed in cells mock infected or treated with the noninvasive strain BS176 (Fig. 4). The total NTP level in BS176-treated cells was slightly elevated at time zero and then returned to levels observed in control cells (Fig. 4). Immediately after infection, we observed lower total NTP levels in M90T-treated cells than in cells treated with the noninvasive strain BS176. By 3 h, total NTP levels in infected cells dropped to 30% of the levels observed in uninfected cells. These results suggest that intracellular bacteria disrupt the energy state of the host cell.

Incorporation of extracellular radiolabeled methionine. It was previously shown that protein synthesis in HeLa cells (12) or murine macrophage cells (7) is severely reduced after infection with *S. flexneri*. To confirm these results, HeLa cells treated with strain BS176 or strain M90T were washed at 1-h intervals postinfection and then overlaid for 20 min with methionine-free MEM supplemented with L-[^{35}S]methionine as described in Materials and Methods. We then measured the incorporation of radioactivity into the total cell fraction, which includes both host cell protein and bacterium-derived protein, or the detergent-soluble TCA-precipitable fraction, which was previously shown to contain primarily but not exclusively host-derived cytosolic macromolecules (12). We found that for as long as 3 h postinfection, M90T-infected cells incorporated extracellular methionine into the total or detergent-soluble fraction to levels slightly less than in BS176-treated cells. However, by 4 h postinfection, M90T-infected cells accumulated less than 40% of the radioactivity into either the total or soluble cell fractions compared with BS176-treated cells (Fig. 5). Similar kinetics were observed previously (12) and suggest that by 4 h postinfection, there is a severe inhibition of host methionine uptake and/or incorporation.

Glucose uptake in infected HeLa cells. Increased hexose transport is a characteristic of cells undergoing metabolic stress and a response to infection with certain transforming viruses (10, 38, 42). To determine whether *S. flexneri*-infected HeLa cells show a similar phenotype, HeLa cells were treated with BS176 or M90T and at 1-h intervals postinfection were overlaid for 20 min with glucose-free MEM supplemented with D-[^{3}H]glucose. We then measured the total amount of radioactivity associated with these cells (Fig. 6) and compared these

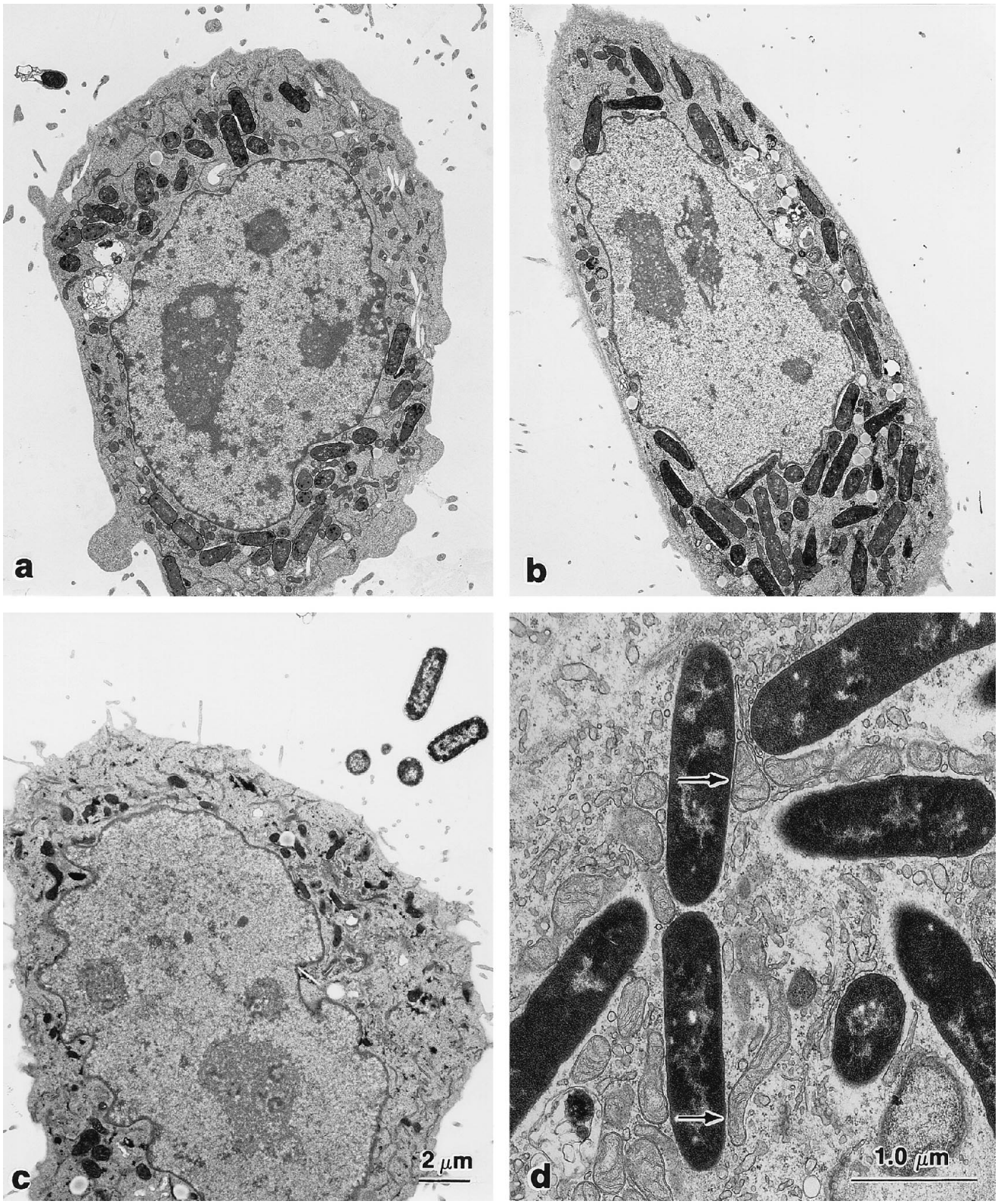

FIG. 3. Ultrastructural morphology of HeLa cells infected with *S. flexneri* M90T. Shown are representative transmission electron micrographs prepared as described in Materials and Methods of HeLa cells at 3 h after infection with the invasive *S. flexneri* strain, M90T (a and b), or the noninvasive strain, BS176 (c). Intracellular bacteria were often observed in close proximity to mitochondria (d). Bars indicate scale.

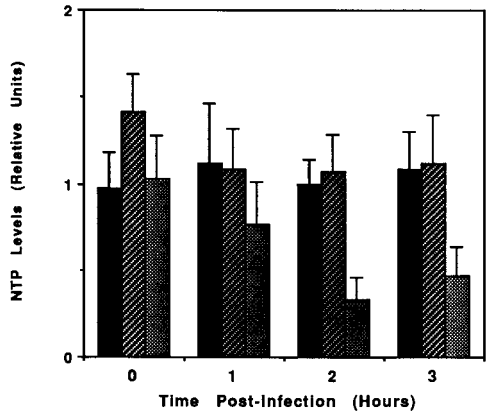

FIG. 4. Total NTP levels in HeLa cells infected with *S. flexneri*. Total NTP levels in HeLa cells (2×10^5) were measured at indicated times postinfection. Treatments: none (solid bars); BS176 (striped bars); M90T (shaded bars). The results represent the averages and standard errors of at least four independent experiments; 1 U represents approximately 1 μmol of NTP.

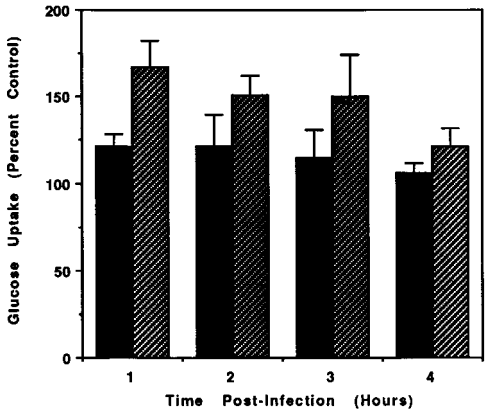

FIG. 6. D-[^{3}H]glucose accumulation into HeLa cells infected with *S. flexneri*. HeLa cells were treated with invasive strain M90T (striped bars) or noninvasive strain BS176 (solid bars) as described in Materials and Methods. Twenty minutes prior to each indicated time point postinfection, HeLa cells were overlaid with glucose-free MEM supplemented with D-[6-^{3}H]glucose. At indicated times, cells were washed and assayed for total radioactivity. Results are presented as percent uptake of control cells (i.e., MEM only). Shown are the averages and standard deviations of three independent experiments done in triplicate.

values with those obtained from control cells. Between 1 and 3 h postinfection, we observed a slightly elevated level of radioactivity associated with HeLa cells treated with BS176 (Fig. 6). We observed a much greater accumulation of radioactivity in HeLa cells after infection with M90T for the entire 4-h experiment (Fig. 6). These results suggest that glucose uptake (e.g., transport) may be induced in HeLa cells after *S. flexneri* infection.

Lactate levels in infected HeLa cells. An increase in the intracellular lactate levels in mammalian cells is indicative of anaerobic metabolism. To test whether HeLa cells infected with *S. flexneri* shift from an aerobic to an anaerobic metabolism, we measured total lactate levels in HeLa cells at 1, 2, 3, or 4 h after treatment with the noninvasive strain, BS176, or the invasive strain, M90T, and compared these levels with those in mock-infected HeLa cells. We detected approximately 75 ± 6 μg of lactate per 5×10^5 HeLa cells. There was no significant increase in this level after as long as 4 h postinfection with M90T (70 ± 7 μg of lactate per 5×10^5 HeLa cells) or BS176 (75 ± 3 μg of lactate per 5×10^5 HeLa cells).

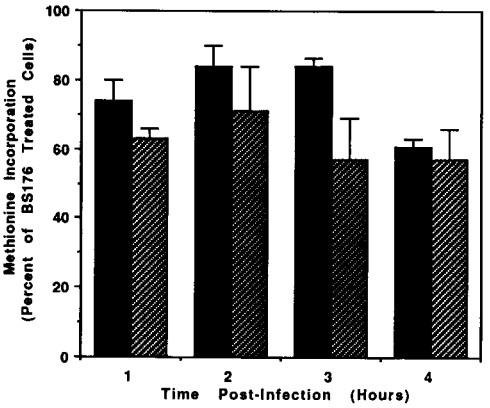

FIG. 5. Accumulation of L-[^{35}S]methionine into HeLa cells infected with *S. flexneri*. HeLa cells were treated with invasive strain M90T or noninvasive strain BS176 as described in Materials and Methods. Twenty minutes prior to each indicated time point postinfection, HeLa cells were overlaid in methionine-free MEM containing 1 μCi of L-[^{35}S]methionine per ml. At indicated times, cells were washed and lysed with deoxycholate. A fraction of cell lysate (0.2 ml) was assayed immediately for radioactivity (total activity). The remaining fraction (0.8 ml) was cleared by microcentrifugation and precipitated with TCA, and the pellet was resuspended in water and assayed for radioactivity; this was defined as soluble activity. The amount of methionine accumulation into the total fraction (solid bars) or the detergent-soluble TCA-precipitable fraction (striped bars) of M90T-treated cells is expressed as the percentage of activity observed in BS176-treated cells, calculated as described in Materials and Methods. Shown are the averages and standard errors from three independent experiments, each done in triplicate.

***hsp70* expression in HeLa cells after *S. flexneri* infection.** The mammalian *hsp70* gene is induced in cells after treatment with conditions that block cellular metabolism, including treatment with specific metabolic inhibitors and temperature upshift, and during infection with certain transforming viruses (42). Therefore, it was of interest to test whether expression of the *hsp70* gene is stimulated after *S. flexneri* infection. We measured *hsp70* mRNA levels in HeLa cells after treatment with BS176 or M90T. Total RNA was isolated from HeLa cells at various times after infection with *S. flexneri* or after a shift to 44°C and then reverse transcribed into cDNA which was used as template in a PCR with primers specific for the human *hsp70* gene (18, 36). As expected, we observed a marked increase in the amount of PCR product obtained from cDNA generated from HeLa cells incubated for 2 h at 44°C compared with the amount present in cells maintained at 37°C (Fig. 7). Surprisingly, there was no detectable difference in the amount of PCR product obtained from cDNA generated from HeLa cells infected with the invasive strain, M90T, compared with the noninvasive strain, BS176. Although at early time points postinfection (i.e., 20 min to 1 h), the amount of PCR product obtained from both treatments was slightly higher than the amount obtained from control cDNA, possibly because of the presence of bacterial lipopolysaccharide. HeLa cells infected with M90T were capable of de novo transcription of the *hsp70* gene because we detected an increase in the amount of PCR product obtained from cDNA generated from HeLa cells which were first infected with M90T and then shifted to 44°C for 1 h. We also observed protein synthesis patterns in infected cells by pulse-labeling with radiolabeled methionine and compared this pattern with that of cells grown at 37°C or those heat-shocked for 1 h. We observed the synthesis of a band corresponding to a molecular mass of 70 kDa in heat-shocked cells but not in M90T-infected cells (data not shown).

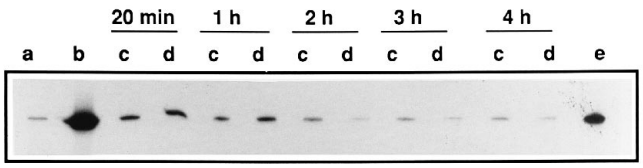

FIG. 7. *hsp70* mRNA levels in HeLa cells following *S. flexneri* infection. HeLa cells (2×10^5) grown at 37°C were treated with BS176 or M90T as described in Materials and Methods or shifted to 44°C. At times posttreatment, total RNA was extracted and reverse transcribed into cDNA by using random primers and Moloney murine leukemia virus reverse transcriptase. cDNA was then used as a template for PCRs in the presence of [^{32}P]dCTP and oligonucleotide primers specific for the human *hsp70* gene; PCR products were then size fractionated on a standard DNA sequencing gel and visualized by exposure to X-ray film. Treatments: none (lane a); heat shock (44°C) for 2 h (lane b); BS176 for indicated times (lanes c); M90T for indicated times (lanes d); M90T and heat shock (44°C) for 1 h (lane e). The amount of *hsp70* PCR product detected from M90T- infected HeLa cells (lanes d) at 2, 3, and 4 h postinfection was slightly less than the amount of product detected from BS176-treated cells at the same time points (lanes c). It is unclear from these experiments whether this difference reflects a difference in stability or production of *hsp70* mRNA in M90T-infected cells compared with BS176-treated cells.

We also used these same cDNA samples as templates for a PCR with oligonucleotide primers specific for the *grp78* gene, an *hsp70* homolog which is induced in response to various stress stimuli, including inhibitors of glycoprotein processing, heavy metals, and amino acid analogs, but not heat shock (37, 41). We found that there was no detectable difference in the amount of PCR product obtained from cDNA synthesized from total RNA purified from M90T-infected HeLa cells compared with RNA purified from uninfected control cells (data not shown).

DISCUSSION

During infection of the human colon, *S. flexneri* encounters several different host cell types, including professional macrophages and intestinal epithelial cells. *S. flexneri* is capable of gaining access to the cytoplasm of both of these cell types, although the outcomes of the infections are dramatically different. In vitro, *S. flexneri*-infected macrophages rapidly lose cell viability and lyse via a process that resembles apoptosis (32, 45). This cytotoxicity is not simply a result of bacterial invasion of the cytoplasm. Rather, it is dependent on the expression of the plasmid-encoded invasin IpaB (44), although the mechanism by which IpaB acts is unknown. In this study we examined the viability and morphology of HeLa cells, a continuous human epithelial cell line, after infection with *S. flexneri*. We found that *S. flexneri*-infected HeLa cells remained viable and intact (i.e., no detectable detachment or lysis [Fig. 2]) for the 4-h duration of the experiments, despite the fact that many cells were heavily infected (Fig. 1 and 3). Except for the presence of numerous electron-translucent vacuoles, *S. flexneri*-infected HeLa cells appeared normal (Fig. 3) and did not show any characteristics of cells undergoing apoptosis, i.e., membrane blebbing or perinuclear chromatin aggregation or DNA fragmentation (data not shown). Therefore, at least within these two cell types, *S. flexneri* is selectively cytotoxic for macrophages. This selective cytotoxicity is reminiscent of the action of lethal toxin of *Bacillus anthracis*, which rapidly kills macrophage cells but not other cell types (14).

Results from experimental animal models of shigellosis are consistent with the hypothesis that *S. flexneri* rapidly kills macrophages but not epithelial cells (22, 29). First, large numbers of killed macrophages are observed in the lamina propria of intestinal villi (22), and individual infected cells show apoptotic morphology (29). While epithelial cells are observed to undergo necrotic-like death during shigellosis, this killing is apparently due to the action of the host inflammatory response rather than intracellular bacteria per se. When polymorphonuclear cells were inhibited from infiltrating the intestine of experimentally infected rabbits, there was no detectable tissue destruction or epithelial cell killing, although there were significant numbers of epithelial cells which contained intracellular bacteria (29).

Intracellular growth is metabolically stressful to the host cell. Although HeLa cells were found to remain viable after infection, we hypothesized that the presence of intracellular bacteria could dramatically affect host cell metabolism and thus be stressful to the host cell. In agreement with this hypothesis, *S. flexneri*-infected HeLa cells showed several biochemical characteristics of cells undergoing metabolic stress (42), including a decrease in total dNTP levels (Fig. 4), reduced ability to incorporate extracellular amino acids (Fig. 5), and increased ability to accumulate extracellular hexoses (Fig. 6). Admittedly, one caveat to these observations is that these biochemical phenotypes are diagnostic characteristics of cells undergoing metabolic stress and do not necessarily provide an insight as to the cause or source of the stress(es). However, it was proposed that the mitochondria may be an early, or even the initial, mediator of the biochemical changes that occur after heat shock or after treatment with metabolic inhibitors (42). Interestingly, *S. flexneri* is often found in close proximity to this organelle (Fig. 3) (6), and it was reported that in a cell-free system, lipid A of *Shigella sonnei* alters mitochondrial respiration (24). An association between mitochondria and intracellular, membrane-bound *Legionella pneumophila* has been previously noted (17).

The reduction of NTP levels in infected cells could be a consequence of altered host cell metabolism, a general stress response, or a direct scavenging of these molecules by intracellular bacteria. Unfortunately, even in the well-studied heat shock response, the factor responsible for the dramatic drop in ATP levels remains unknown. It was recently reported that *S. flexneri* expresses a plasmid-encoded, periplasmically located apyrase activity which nonspecifically hydrolyses NTPs (5). It is possible that this activity contributes to the reduction in NTPs that we observed in infected cells. We are currently constructing a strain with a mutation in the gene encoding this enzyme to test this hypothesis (8a). The intracellular parasite *Toxoplasma gondii* is a purine auxotroph which secretes a potent NTP hydrolase which has shown to function within host cells (3).

In agreement with the study by Hale and Formal (12), we found that *S. flexneri*-infected HeLa cells accumulated less extracellular radiolabeled amino acids into both the total and detergent-soluble TCA-precipitable cell fractions compared with HeLa cells which had been treated with the noninvasive *S. flexneri* strain, BS176 (Fig. 5). It was previously hypothesized that the production of Shiga toxin may be responsible for this inhibition (12). This is unlikely in the case of M90T-infected HeLa cells because (i) this strain does not express significant Shiga toxin activity or react positively with a probe against SLT-1 (11, 33) and (ii) Shiga-toxin induced cytotoxicity occurs on a time scale of between 8 and 24 h (12), not between 1 and 4 h (Fig. 5). While it is clear that *S. flexneri* can efficiently scavenge and incorporate amino acids from the host cytosol into its own proteins (12, 15), it is not known whether this property is involved in virulence.

The decreased ability of M90T-infected HeLa cells to accumulate extracellular methionine correlated with an increased ability to accumulate extracellular glucose (Fig. 6). It is un-

likely that this increase in glucose accumulation is simply a result the loss of membrane permeability in M90T-infected cells, since neither methionine nor trypan blue accumulation increased under these conditions (Fig. 5). These results indicate that selective cellular transport processes may be maintained in heavily infected cells. It is interesting that a similar inverse correlation between protein synthesis and glucose transport was observed in BHK-21 cells infected with cytolytic, nontransforming viruses such vesicular stomatitis virus, Semliki Forest virus, and herpes simplex virus (10). This phenotype (i) correlated with intracellular virus production, (ii) was genetically separable, in the case of herpes simplex virus infection, from the induction of the heat shock proteins, and (iii) and reflected an increased rate of transport across the plasma membrane.

It was surprising to discover that there was no detectable increase in expression of the *hsp70* gene in HeLa cells following infection with *S. flexneri*, since this stress-inducible gene has been shown to be activated in host cells infected by other intracellular bacterial pathogens, including *Listeria monocytogenes* (35) and *Mycobacterium leprae* (27). Our result supports the hypothesis that the induction of *hsp70* expression is highly pathogen specific and is not simply a result of cellular violation. Whereas infection of HeLa cells with the DNA viruses adenovirus type 5 and herpes simplex virus type 1 stimulated expression of the *hsp70* gene, infection with vaccinia virus had no effect (30). It is likely that *S. flexneri* avoids inducing the *hsp70* gene, rather than blocking its expression per se, since we were able to detect induction of *hsp70* in *S. flexneri*-infected cells which had also been heat shocked (Fig. 7). Preliminary experiments suggest there is little change in the profile of host protein synthesis in cells infected with *S. flexneri* (21a).

The ability of *S. flexneri* to penetrate and multiply within the cytosol of intestinal epithelial cells is essential for disease production (20). Our finding that *S. flexneri* can rapidly multiply to high numbers within the cytoplasm of epithelial cells without inducing one of the major stress response pathways, or inducing host detachment or lysis, suggests that this bacterium has efficient stealth-like means to exploit the host cell environment. The observation that infected HeLa cells are altered in at least three biochemical parameters indicates that intracellular *S. flexneri* does not go unnoticed. It is hypothesized that *S. flexneri* may initially invade the specialized M cells of the follicle-associated epithelium overlying lymphoid tissue of the colon (29, 39), an area rich in professional macrophage cells. In vivo, it may be advantageous for the bacterium to escape from and destroy resident macrophage cells which possess numerous antibacterial activities. On the other hand, the bacterium may wish to prolong its life within the intracellular compartment of epithelial cells, as it is an environment condusive for bacterial growth (at least within in vitro-grown cells) and protected from circulating immune functions. These results provide a basis on which to pursue more detailed mechanistic studies into the undoubtedly intricate metabolic interaction that occurs between the epithelial host cell and the intracellular pathogen.

ACKNOWLEDGMENTS

We thank W. Beatty and L. Bernardini for critical review of the manuscript and P. Gounon and B. Demers for help with electron microscopy.

N.M. gratefully acknowledges financial support from the Mrs. Frank Howard Postdoctoral Fellowship from the Institut Pasteur, a Fulbright Scholar Research Award from the Council for the International Exchange of Scholars (Washington, D.C.), and the Philippe Foundation (Paris, France).

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Editor: A. O'Brien