Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4179–4183, June 1990
Microbiology

Differential protein expression by *Shigella flexneri* in intracellular and extracellular environments

(invasion plasmid antigen expression)

VIRGINIA L. HEADLEY AND SHELLEY M. PAYNE

Department of Microbiology, University of Texas, Austin, TX 78712-1095

Communicated by Esmond E. Snell, February 26, 1990

ABSTRACT **Shigellae were intrinsically radiolabeled with [^{35}S]methionine either extracellularly or while multiplying within infected HeLa cell monolayers. A complex pattern of suppression and induction of proteins was observed. Proteins of approximately 97, 62, 58, 50, 25, and 18 kilodaltons (kDa) were induced in *Shigella flexneri* isolated from infected monolayers. Proteins of 100, 85, 70, 64, and 55 kDa were suppressed under the same conditions but were seen in cells labeled in the tissue culture medium alone. Protein expression during the stages of attachment, invasion, and intracellular multiplication was examined by pulse-labeling. The 58-kDa protein was induced only during invasion, and the 62- and 25-kDa proteins were induced only during intracellular multiplication. Shift into a minimal medium with ion concentrations and pH mimicking intracellular conditions and endosomal pH resulted in the induction of the 97- and 58-kDa proteins, and reduction of the intracellular-like medium with 2-mercaptoethanol resulted in the induction of the 97-, 50-, and 25-kDa proteins and suppression of the 55-kDa protein. Radioimmunoprecipitations of shigellae grown *in vitro* and *in vivo* revealed differential expression of immunogenic proteins. Proteins corresponding in size to IpaB (62 kDa), IpaC (42 kDa), and IpaD (38 kDa) were lost during intracellular multiplication, whereas another protein corresponding to IpaA (80 kDa) was found to increase under the same conditions.**

Shigella flexneri is an etiologic agent of human bacillary dysentery, a disease which is characterized by bloody mucous diarrhea. The ulcerative lesions produced in the colon by the shigellae are the result of penetration and multiplication within the epithelial cells of the colonic mucosa, invasion into adjacent cells, and elicitation of an acute inflammatory response (1, 2). Factors involved in these processes have not been fully characterized, but they include O antigen (3) and surface proteins which specifically interact with eukaryotic cell membranes or other structures such as microfilaments (4–7).

Invasion of HeLa cell monolayers by *S. flexneri* has been used as a model which correlates with virulence in this organism, as penetration into epithelial cells is requisite for virulence (1). The HeLa cell model was used in this study to determine whether *S. flexneri* responds differentially to an intracellular habitat as opposed to the extracellular environment in which it is most often studied. Throughout this study, shigellae isolated from infected cells are referred to as grown *in vivo*, and bacteria from media are referred to as grown *in vitro*.

MATERIALS AND METHODS

Bacterial Strain. The wild-type *S. flexneri* 2a strain SA100 (8) was stored at −80°C in Luria broth/20% (vol/vol) glycerol.

Media and Reagents. Bacteria were routinely cultured in Luria broth or on tryptic soy broth (BBL) with 1.5% agar and 0.01% Congo red (Sigma) to monitor the ability of the bacteria to bind the dye (Crb$^+$); loss of this ability correlates with alterations in the virulence-associated large plasmid (9) and loss of virulence (10). For radiolabeling *in vitro*, a minimal medium was formulated to contain ions at concentrations comparable to those found in the eukaryotic cytoplasm. This intracellular salts medium (ISM) contained 170 mM potassium phosphate, 0.5 mM magnesium sulfate, 1 μM calcium chloride, 6 mM potassium sulfate, 5 mM ammonium chloride, 5 mM sodium chloride, 0.4% glucose, and nicotinic acid at 2 μg/ml. When a reducing medium was desired, 2-mercaptoethanol was added to 100 mM. The pH was adjusted by varying the relative concentrations of dibasic and monobasic potassium phosphate. Chemicals for SDS/PAGE were obtained from Bio-Rad. All other chemicals were analytical grade.

[^{35}S]Methionine Labeling of Intracellular Bacteria. HeLa cell monolayers were routinely cultured in Earle's minimal essential medium + 2 mM glutamine + 10% fetal calf serum (EMEM). Subconfluent HeLa cell monolayers were infected by using a modification of the procedure of Hale and Formal (11). Monolayers were washed in PBS (26.5 mM potassium chloride/137 mM sodium chloride/1.5 mM monobasic potassium phosphate/8 mM dibasic sodium phosphate, pH 7.5), overlaid with fresh medium, and incubated in a 5% CO_2 atmosphere at 37°C for 2 hr prior to infection. Crb$^+$ SA100 were grown for 4 hr at 37°C in Luria broth, pelleted, and resuspended in EMEM. Bacteria were then added to the monolayers to give 2×10^8 colony-forming units/ml. To allow for attachment and invasion, the monolayers were centrifuged at 1700 × *g* for 10 min at 20°C and incubated at 37°C in 5% CO_2 for 1.5 hr. The medium was removed, the monolayers were washed four times in PBS, and fresh EMEM lacking fetal calf serum and methionine and containing gentamicin at 16 μg/ml and 200 μCi of [^{35}S]methionine (Tran^{35}S-Label, ICN; 1 Ci = 37 GBq) was added. For pulse-labeling experiments, 500 μCi of [^{35}S]methionine was added. In studies of the effect of cycloheximide (Sigma), the inhibitor was added to 75 μg/ml. The monolayers were incubated in the presence of label at 37°C in 5% CO_2. HeLa cells were then harvested by trypsinization or, when designated for radioimmunoprecipitation analysis, were harvested with Enzyme Free Cell Dissociation Solution (Specialty Media, Lavalette, NJ) according to the supplier's protocol. Cells were pelleted and washed with PBS. The pellets were resuspended three times in 0.5% sodium deoxycholate in PBS to lyse the HeLa cells. The remaining bacterial cells were washed in PBS, resuspended in TM buffer (10 mM Tris·HCl, pH 7.4/5 mM $MgCl_2$) to 0.5–2 × 10^5 cpm of trichloroacetic acid-precipitable material per μl, and stored at −80°C until needed. When desired, Chinese hamster ovary cells, strain

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: Crb$^+$, Congo red-binding.

K1, were substituted for HeLa cell monolayers without any change in protocol or effect.

[^{35}S]Methionine Labeling of Extracellular Bacteria.

EMEM. Crb$^+$ SA100 were incubated for 4 hr at 37°C in Luria broth, pelleted, and resuspended in 1 vol of EMEM lacking fetal calf serum and methionine. Resuspended bacteria were incubated at 37°C for 15 min and [^{35}S]methionine was added, 25 μCi for 1-hr labeling or 50 μCi when a 20-min labeling was indicated. Radiolabeled bacteria were pelleted and resuspended in TM buffer to 0.5–2 × 10^5 cpm of acid-precipitable material per μl.

ISM. Crb$^+$ SA100 were grown for 4 hr at 37°C in Luria broth, pelleted, washed, and resuspended in 1 vol of ISM (the intracellular salts medium) at 37°C. Suspensions were incubated for 15 min, 25–50 μCi of [^{35}S]methionine was added, and incubation was continued for 1 hr at 37°C. Radiolabeled bacteria were pelleted and resuspended in TM buffer to 2 × 10^5 cpm of acid-precipitable material per μl.

SDS/PAGE. Samples of radiolabeled bacteria (0.5–2 × 10^5 cpm) were solubilized and proteins were separated by one-dimensional SDS/PAGE (12) using 12% (wt/vol) acrylamide. The gels were dried and exposed to Kodak X-Omat-AR film at room temperature.

Radioimmunoprecipitations. Radiolabeled *Shigella* proteins were immunoprecipitated with convalescent monkey polyvalent antiserum (13) or monoclonal antibodies directed against IpaB or IpaC (14) and staphylococcal protein A-Sepharose CL-4B (Pharmacia) in 150 mM NaCl/1 mM EDTA/50 mM Tris·HCl, pH 7.5/0.5% Triton X-100/0.1% SDS. Immunoprecipitated proteins were solubilized and separated by one-dimensional SDS/PAGE as above.

RESULTS

Protein Synthesis by *S. flexneri in Vitro* and *in Vivo*. To study protein synthesis by shigellae during intracellular multiplication, infected monolayers were radiolabeled with [^{35}S]-methionine for 1 hr. The radiolabeled bacteria were isolated, and proteins were separated by SDS/PAGE and quantitated by densitometry of autoradiograms. Fig. 1 shows a representative autoradiogram of the protein profiles obtained. Although the *in vivo* bacterial protein profiles (lanes 2 and 4) appear similar to the *in vitro* profiles (lanes 3 and 5) and do not appear to contain appreciable amounts of HeLa cell proteins (lane 1), comparison of *in vivo* and *in vitro* profiles indicates a complex pattern of suppression and induction of proteins. Proteins of 97, 62, and 50 kDa were detected *in vitro* but had significantly more label incorporated *in vivo*. Proteins of 58, 25, and 18 kDa had lower label incorporation *in vivo* than the three proteins just mentioned; however, there appears to be no label incorporated into proteins of these sizes *in vitro*. In contrast, there were proteins which appeared to be suppressed *in vivo*, but which had a significant amount of label incorporated *in vitro*: proteins of >200, 110, 100, 85, 70, 64, and 55 kDa. The response was specific to interaction with cell monolayers and not due to some artifact of manipulation. Bacteria that were mock-infected onto tissue culture dishes in the absence of cell monolayers did not produce the induction/suppression profile observed in bacteria grown *in vivo* (data not shown).

Effect of Cycloheximide on Induction and Suppression of Proteins *in Vivo*. Hale and Formal (11) have shown that HeLa cells infected with *S. flexneri* do not incorporate radioactive amino acids into protein, presumably due to action by a *Shigella* cytotoxin. To ensure that host protein synthesis was not producing any of the induced proteins, infected and noninfected monolayers were radiolabeled in the presence of cycloheximide (Fig. 2). Noninfected HeLa cells did not incorporate any [^{35}S]methionine into proteins in the presence of cycloheximide (lane 2). Cycloheximide had no effect on shigellae labeled *in vitro*. Protein profiles of shigellae isolated from infected cells were also identical in the presence and absence of cycloheximide, indicating that none of the bands observed on the autoradiograms were due to host protein synthesis. Therefore, cycloheximide was not added during any subsequent infections.

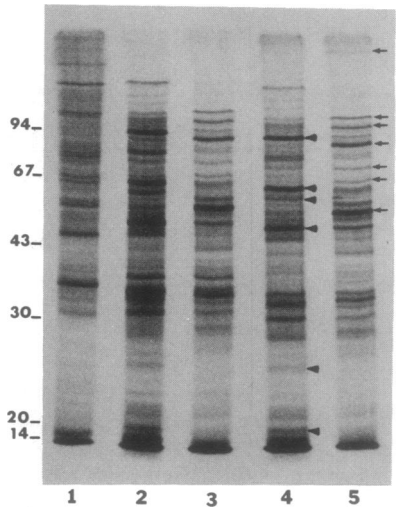

FIG. 1. *S. flexneri* labeling *in vitro* and in HeLa cell monolayers. Shigellae were radiolabeled with [^{35}S]methionine for 1 hr extracellularly or while multiplying within HeLa cell monolayers. Lane 1, noninfected HeLa cell proteins; lanes 2 and 4, proteins from shigellae isolated from infected HeLa cell monolayers; lanes 3 and 5, proteins from shigellae radiolabeled in EMEM. Bacterial proteins were solubilized with (lanes 2 and 3) or without (lanes 4 and 5) addition of the reducing agent 2-mercaptoethanol. Proteins induced by intracellular multiplication (arrowheads) included proteins of approximately 97, 62, 58, 50, 25, and 18 kDa. Proteins suppressed during intracellular multiplication (small arrows) included proteins >200 (nonreduced only), 110, 100, 85, 70, 64, and 55 kDa. Positions of molecular mass markers are noted to the left in this and the following autoradiograms.

Time Course of Induction and Suppression of Proteins *in Vivo*. To study further the induction and suppression of proteins *in vivo*, infected monolayers were pulse-labeled for

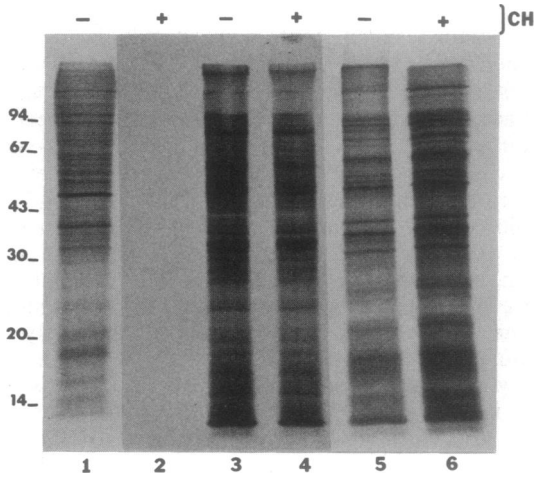

FIG. 2. Effect of cycloheximide (CH) on induction and suppression of proteins *in vivo*. Shigellae were radiolabeled *in vitro* and *in vivo* in the presence or absence of cycloheximide. Lanes 1, 3, and 5 were labeled in the absence of the inhibitor; lanes 2, 4, and 6 were labeled in its presence. Lanes 1 and 2, noninfected HeLa cell proteins. Note: no proteins were synthesized by the HeLa cells in the presence of cycloheximide. Lanes 3 and 4, proteins from shigellae radiolabeled in EMEM. Lanes 5 and 6, proteins from shigellae isolated from infected HeLa cell monolayers.

periods chosen to correspond with the various stages of attachment, invasion, and intracellular multiplication (Fig. 3), as confirmed by observations of stained duplicate monolayers. Under the described conditions, shigellae routinely attached to host cells within 15–30 min, were engulfed by phagocytosis at 30–60 min, were located within the cytoplasm shortly thereafter, and began intracellular multiplication. Multiplication continued for 1–2 hr until the host cells lysed. Fig. 4 shows the pattern of induction and suppression of proteins during these stages, which was confirmed by densitometric analysis (not shown). Lane 2 shows the *in vitro* profile for bacteria labeled for 20 min in EMEM. This profile differs from the profile seen in Fig. 1, lane 3, in which bacteria were labeled for 1 hr in EMEM. Previously, there was a large amount of label incorporated into a 55-kDa protein but a reduced amount of label in a 50-kDa protein. In this autoradiogram, there appears to be equal amounts of label in the two proteins. Shigellae radiolabeled and isolated during the attachment stage of infection induced 97- and 58-kDa proteins (Fig. 4, lane 3, arrowheads) at significantly high levels, whereas 100-, 84-, 70-, and 55-kDa proteins showed significant reduction in the same time period, as compared to bacteria labeled *in vitro* (lane 2). At 1 hr after infection, during the invasion stage (lane 5), a 25-kDa protein (arrowhead) was induced. At this time (Fig. 3, time C), any plates remaining to be radiolabeled were washed and the medium was replaced with one containing gentamicin to kill any remaining extracellular bacteria. A 50-kDa and a 25-kDa protein were consistently expressed *in vivo* after the addition of gentamicin (Fig. 4, lanes 6–9), confirming what was seen in lane 2 of Fig. 1. A 62-kDa protein which was only weakly expressed during attachment and invasion, and moderately expressed after the addition of gentamicin (lane 6), was seen strongly expressed during all remaining intracellular multiplication profiles (lanes 7–9, arrowheads).

Protein Expression in ISM. The environment that a shigella encounters once inside the HeLa cell cytoplasm is quite different from the extracellular environment that it left. One major difference is the concentrations of ions encountered. In EMEM as well as in extracellular bodily fluids, the sodium and calcium ion concentrations are high, but the magnesium and potassium levels are relatively low. In the cytoplasm, the reverse is true. In addition, invading bacteria also encounter a pH shift down after endocytosis, and then another pH shift back to neutral as they enter the cytoplasm. The cytoplasm is also a reducing, rather than an oxidizing, environment. Thus, a potassium phosphate-buffered minimal medium that had ion concentrations mimicking those found intracellularly

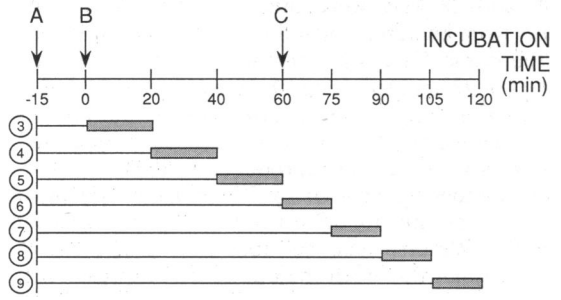

FIG. 3. Pulse-labeling of shigellae during the various stages of infection. Bacteria were centrifuged onto multiple HeLa cell monolayers (time A). After 15 min of incubation to allow attachment, all media were replaced to remove nonassociated bacteria (B). Gentamicin was added to cultures at time C to kill extracellular bacteria. Individual infected monolayers were labeled with 500 μCi of [^{35}S]methionine at 37°C in 5% CO_2 for time periods indicated by shaded boxes and were harvested at the end of the labeling period. Solid lines indicate incubation at 37°C in 5% CO_2. Numbers in circles at left correspond to lane numbers in Fig. 4.

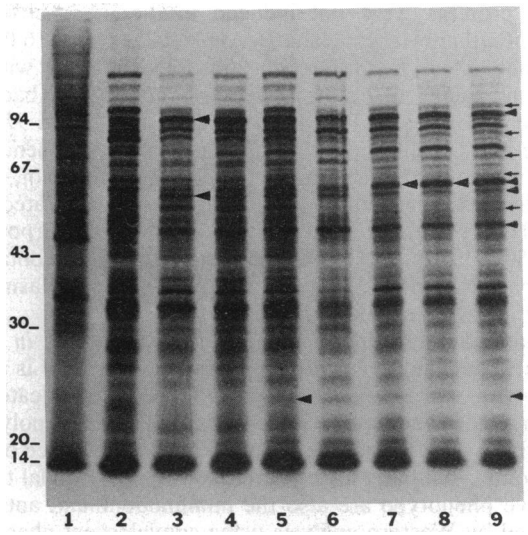

FIG. 4. Time course of induction and suppression of proteins *in vivo*. Shigellae were pulse-labeled during sequential time periods in infected monolayers as indicated in Fig. 3. Lane 1, noninfected HeLa cell proteins; lane 2, proteins from shigellae radiolabeled in EMEM; lane 3, proteins from shigellae radiolabeled during attachment; lanes 4 and 5, proteins from shigellae radiolabeled during early and later invasion, respectively; lanes 6–9, proteins from shigellae radiolabeled during the stages of intracellular multiplication. Proteins induced (arrowheads) included proteins of approximately 97, 62, 58, 50, and 25 kDa. Proteins suppressed (small arrows) included proteins of 100, 85, 70, 64, and 55 kDa.

(ISM) was formulated. By varying the dibasic and monobasic components of ISM, the pH was altered to reflect the various pH values the invading bacteria might encounter. 2-Mercaptoethanol was also added to mimic the reducing condition of the cytoplasm. Bacteria were grown in a complex medium and then shifted and radiolabeled in these various conditions to determine whether the suppression and induction of proteins in *Shigella* were due to the change in ionic environment. Fig. 5 shows the autoradiogram of the resulting protein profiles. At pH 4.8, probably the lowest pH that the bacterium might encounter in the endosome, in the absence of reducing agent in the medium, there was increased incorporation of label into proteins of 97 and 58 kDa (lane 1), as was seen in the first 20 min after infection (Fig. 4, lane 3).

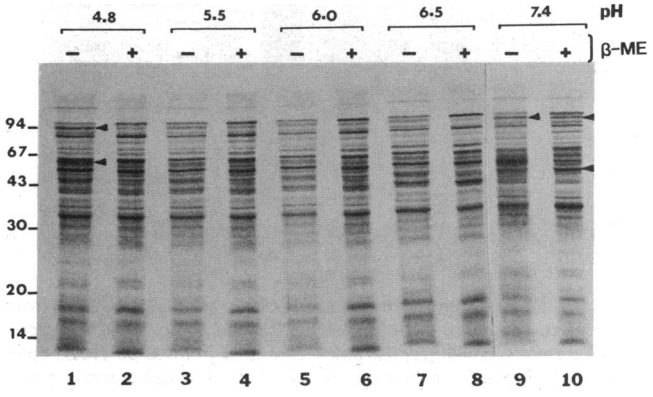

FIG. 5. Effect of pH and 2-mercaptoethanol (β-ME) on protein expression in ISM. Shigellae were radiolabeled at pH 4.8 (lanes 1 and 2), pH 5.5 (lanes 3 and 4), pH 6.0 (lanes 5 and 6), pH 6.5 (lanes 7 and 8), and pH 7.4 (lanes 9 and 10) in the absence (lanes 1, 3, 5, 7, and 9) and presence of 100 mM 2-mercaptoethanol (lanes 2, 4, 6, 8, and 10). Proteins induced (arrowheads) by shift to nonreducing ISM at pH 4.8 included proteins of approximately 97 and 58 kDa. The 97-kDa protein was induced by shift to ISM at pH 7.4, and a 50-kDa protein was induced in the presence of 2-mercaptoethanol.

These proteins were not induced in the presence of 2-mercaptoethanol (Fig. 5, lane 2). At pH values of 5.5, 6.0, and 6.4, there were no differences observed with or without reducing agent, or from profiles obtained from bacteria labeled in EMEM (pH 7.4). At pH 7.4 in ISM, a 97-kDa protein was seen in both the presence and absence of reducing agent. In the presence of 2-mercaptoethanol, there was also a decrease in the amount of label incorporated into a 55-kDa protein and an increase in the amount incorporated into a 50-kDa protein. This is similar to those results obtained from bacteria isolated after invasion into the cytoplasm and during intracellular multiplication.

Radioimmunoprecipitations of *S. flexneri* Grown *in Vitro* and *in Vivo*. The invasive phenotype in *S. flexneri* is associated with expression of a conserved set of genes located on the large virulence-associated plasmid (15). Four polypeptides (IpaA, IpaB, IpaC, and IpaD) which are encoded on the invasion plasmid and have been found to be essential to the invasive phenotype are also the immunodominant antigens detected by Western analysis using convalescent-phase antiserum (13). Convalescent-phase antiserum from a monkey was used to immunoprecipitate any immunologically significant proteins into which label had been incorporated during the various stages *in vivo*, as well as *in vitro* (Fig. 6). Immunoprecipitations of shigellae radiolabeled in EMEM detected proteins which correlated in size to all four major immunogenic proteins: IpaA (80 kDa), IpaB (62 kDa), IpaC (42 kDa), and IpaD (38 kDa). In all *in vitro* preparations, IpaB and IpaC were the immunodominant antigens; however, immunoprecipitation detected more proteins than are usually detected by standard Western analysis. Shigellae isolated during attachment and invasion from HeLa cell monolayers that had been harvested with trypsin were found to have lost IpaB, -C, and -D, presumably due to the action of the protease (data not shown). When an EDTA-based solution was substituted for trypsin to harvest the monolayers, the antigens were detectable, but only in immunoprecipitations of shigellae isolated during attachment (lanes 2 and 6) and early stages of invasion (lanes 3 and 7). Immunoprecipitates of shigellae during intracellular multiplication did not contain these three antigens (lanes 4, 5, and 8). However, immunoprecipitates from a late infection of HeLa cell monolayers did contain detectable, but still reduced, levels of IpaB (lane 9). Proteins of 140, 100, 87, 80, 68, and 28 kDa were immunoprecipitated in shigellae isolated *in vivo*, but either not at all or in reduced amounts in shigellae which were radiolabeled in EMEM (lane 1). A protein of 80 kDa, which corresponds in size to IpaA, was immunoprecipitated from all preparations; however, it was immunoprecipitated in increasing quantities with time of intracellular multiplication. The 68-kDa protein was immunoprecipitated only from preparations isolated during intracellular multiplication in HeLa cells (lanes 8 and 9).

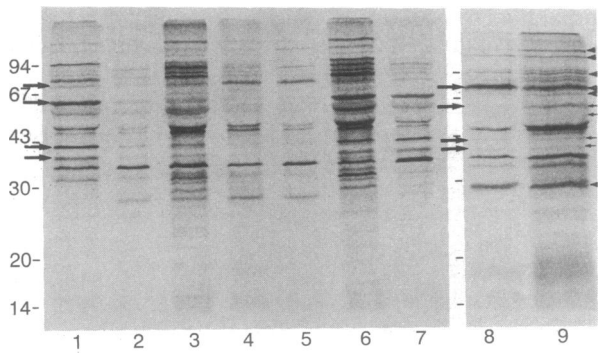

FIG. 6. Radioimmunoprecipitations of *S. flexneri* grown *in vitro* and *in vivo*. Shigellae radiolabeled in EMEM (lane 1), in infected monolayers of CHO-K1 cells (lanes 2–5), and in infected HeLa cell monolayers (lanes 6–9) were immunoprecipitated with convalescent monkey antiserum. Bacteria were isolated 20 min post infection (p.i.) during attachment (lanes 2 and 6), 40 min p.i. during invasion (lanes 3 and 7), 60 min p.i. during the onset of intracellular multiplication (lanes 4 and 8), and 120 min p.i. during late intracellular multiplication (lanes 5 and 9). Proteins corresponding in size to IpaA (80 kDa), IpaB (62 kDa), IpaC (42 kDa), and IpaD (38 kDa) are noted to the left of each autoradiogram with large arrows. Proteins induced during infection (arrowheads to the right) included proteins of 140, 100, 87, 80, 68, and 28 kDa, and proteins lost during infection (small arrows to the right) included proteins of 62, 55, 42, and 38 kDa.

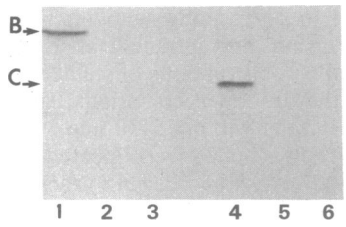

FIG. 7. Radioimmunoprecipitations of invasion plasmid antigens B and C (IpaB and IpaC) of *S. flexneri*. Shigellae radiolabeled in EMEM (lanes 1 and 4) and in infected monolayers of CHO-K1 cells (lanes 2, 3, 5, and 6) were immunoprecipitated with monoclonal antibody 2F1, which is directed against IpaB (lanes 1–3), or 2G2, which is directed against IpaC (lanes 4–6). Bacteria were isolated 40 min after infection during invasion (lanes 2 and 5) and 60 min after infection during the onset of intracellular multiplication (lanes 3 and 6).

To confirm that the antigens lost during the infection process were indeed invasion plasmid antigens, monoclonal antibodies directed against IpaB and IpaC were used to immunoprecipitate Shigella proteins produced *in vitro* and during invasion and intracellular multiplication in CHO-K1 cells (Fig. 7). IpaB and -C were immunoprecipitated only from extracellular bacteria (lanes 1 and 4). The antigens were not detected in shigellae isolated during invasion and intracellular multiplication, indicating that active synthesis of these proteins no longer occurred during these stages of infection.

DISCUSSION

It is apparent from the results presented here that differential protein expression is occurring in *S. flexneri* during invasion of eukaryotic cells. *In vivo*, proteins of 100, 85, 70, 64, and 55 kDa were suppressed and proteins of 97, 62, 58, 50, 25, and 18 kDa were induced. Immunogenic proteins of 140, 100, 87, 80, 68, and 28 kDa were immunoprecipitable in greater amounts from bacteria isolated *in vivo*. Bacteria shifted into ISM, the intracellular salts medium, at low pH induced the synthesis of 97- and 58-kDa proteins, and at neutral pH in the presence of a reducing agent also induced 50- and 25-kDa proteins. However, the entire pattern of suppression and induction *in vivo* was not reproduced in ISM.

An immunogenic protein of 140 kDa (VirG) has been shown to be essential for cell-to-cell spread of shigellae in cell culture (7). Western analysis does not always detect this protein (13), but this might be due to low expression by the shigellae. Immunoprecipitations indicated a higher expression of a 140-kDa protein during intracellular multiplication as compared to earlier stages of infection or growth *in vitro*. In addition to the 140-kDa protein, a protein with a molecular mass corresponding to IpaA (80 kDa) was also immunoprecipitated in greater amounts from intracellular shigellae. The role of IpaA in pathogenesis has yet to be determined, and Tn*5* insertions into *ipaA* have been isolated which do not affect virulence (16). An increase in immunoprecipitable amounts of IpaA during intracellular multiplication could

indicate an intracellular role for this protein rather than a role in invasion, thus possibly explaining the coordinate regulation of *ipaA* with other factors required for virulence. Surprisingly, the other invasion plasmid antigens, IpaB, -C, and -D, were not detected in immunoprecipitates of shigellae isolated *in vivo*. Since only newly synthesized proteins are detected by this procedure, the loss of these antigens would suggest that their role in virulence occurs early in the infection process, and once inside the host cell, their active synthesis is no longer required and is repressed.

In a similar system, Finlay *et al.* (17) were able to show that *Salmonella* induced the synthesis of several new proteins during adherence to fixed epithelial cell monolayers. Mutants unable to synthesize the induced proteins also were unable to adhere to and invade cell monolayers, thus implying a role for these proteins in pathogenicity. The exact role of the induced proteins in adherence or invasion was not elucidated. Similarly, in data presented in this paper, two proteins, 97 and 58 kDa, were induced during the attachment stage of *Shigella* infection of HeLa cell monolayers. However, the 97- and 58-kDa proteins could be induced *in vitro* by altering the ion concentrations and lowering the pH without generating the entire suppression/induction profile seen *in vivo*. The complete regulation of the response in shigellae remains to be determined.

Microorganisms have been shown to possess various operons which are regulated by a number of environmental signals (18), and this regulation allows for the adaptation by these bacteria to various environmental conditions. Often, virulence factors are expressed only under environmental conditions which might be found in the host such as elevated temperature (37°C), low iron availability, and, for intracellular pathogens, the interior environment of the eukaryotic cell (19). Environmental regulation of virulence factors includes temperature regulation of fimbriae expression in *Escherichia coli* (20) and penetration into epithelial cell monolayers in *Shigella* (21) and osmotic regulation of cholera toxin (22) and pili production in *Vibrio cholerae* (23). The ion concentration difference between the extracellular and intracellular environments has been shown to be significant in the pathogenesis of diseases caused by *Yersinia* (24, 25) and is thought to play a role in the regulation of the onset of intracellular multiplication in *Rickettsia* (19). *Coxiella burnetti*, an obligate intracellular pathogen, becomes metabolically active only upon exposure to a low pH which corresponds to the intraendosomal environment (26). Chlamydiae possess an outer membrane protein rich in disulfide bonds that is reduced by the intracellular environment, and this step is presumed to be necessary in order for the chlamydiae to differentiate into their metabolically active form (27, 28). Temperature regulation does not constitute the entire environmental response of shigellae, as data in this paper indicate. The *in vitro* results obtained in ISM at low pH or in the presence of a reducing agent seem to indicate that, *in vivo*, *S. flexneri* could be responding to a cascade of events, rather than a single intracellular signal such as low calcium or high potassium concentration.

Major *Shigella* antigens have been identified in the past by using Western blot analysis of shigellae grown in standard complex growth media. Shigellae isolated from infected HeLa cell monolayers were not of sufficient quantity for standard Western blot analysis; however, the fact that proteins produced by shigellae isolated from infected cells are immunoprecipitable by convalescent antiserum indicates that this response occurs in and is recognized by the host, and it further substantiates the use of the HeLa cell invasion model as an *in vivo* model system. The study presented here also indicates that until the intracellular habitat can be reconstructed *in vitro*, the entire *in vivo* story of *S. flexneri* may not be fully realized.

Adaptation to the specific intracellular environment is an important evolutionary step for all intracellular pathogens. The differential protein expression by shigellae presented here indicates that it has a specific response to this particular habitat. Because virulence in shigellae is multifactorial, understanding of the role specific proteins play has not yet been achieved. Reconstruction of the environment *in vivo* will allow elucidation at the protein level of the effects of specific mutations which eliminate virulence, and it will also facilitate studies of the regulation of virulence in *S. flexneri*.

We thank Edwin Oaks for the gracious gift of monkey antiserum and monoclonal antibodies, Dennis Brown and Judith Edwards for their assistance in tissue culture preparation, and Nathan Davis for assistance with immunoprecipitation analysis. This study was supported by Grant AI 16935 from the National Institutes of Health and Grant DMB-8819169 from the National Science Foundation. V.L.H. was supported by a predoctoral fellowship from the National Science Foundation.

1. LaBrec, E. H., Schneider, H., Magnani, T. J. & Formal, S. B. (1964) *J. Bacteriol.* **88,** 1503–1518.
2. Formal, S. B., Hale, T. L. & Sansonetti, P. J. (1983) *Rev. Infect. Dis.* **5,** 702–707.
3. Formal, S. B., Gemski, P., Jr., Baron, L. S. & LaBrec, E. H. (1970) *Infect. Immun.* **1,** 279–287.
4. Clerc, P., Baudry, B. & Sansonetti, P. J. (1986) *Ann. Inst. Pasteur/Microbiol.* **137A,** 267–278.
5. Clerc, P. & Sansonetti, P. J. (1987) *Infect. Immun.* **55,** 2681–2688.
6. Daskaleros, P. A. & Payne, S. M. (1987) *Infect. Immun.* **55,** 1393–1398.
7. Pál, T., Newland, J. W., Tall, B. D., Formal, S. B. & Hale, T. L. (1989) *Infect. Immun.* **57,** 477–486.
8. Payne, S. M., Niesel, D. W., Peixotto, S. S. & Lawlor, K. M. (1983) *J. Bacteriol.* **155,** 949–955.
9. Maurelli, A. T., Blackmon, B. & Curtiss, R., III (1984) *Infect. Immun.* **43,** 397–401.
10. Payne, S. M. & Finkelstein, R. A. (1977) *Infect. Immun.* **18,** 94–98.
11. Hale, T. L. & Formal, S. B. (1981) *Infect. Immun.* **32,** 137–144.
12. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
13. Oaks, E. V., Hale, T. L. & Formal, S. B. (1986) *Infect. Immun.* **53,** 57–63.
14. Mills, J. A., Buysse, J. M. & Oaks, E. V. (1988) *Infect. Immun.* **56,** 2933–2941.
15. Baudry, B., Maurelli, A. T., Clerc, P., Sadoff, J. C. & Sansonetti, P. J. (1987) *J. Gen. Microbiol.* **133,** 3403–3413.
16. Sasakawa, C., Kamata, K., Sakai, T., Makino, S., Yamada, M., Okada, N. & Yoshikawa, M. (1988) *J. Bacteriol.* **170,** 2480–2484.
17. Finlay, B. B., Heffron, F. & Falkow, S. (1989) *Science* **243,** 940–943.
18. Stock, J. (1987) *BioEssays* **6,** 199–203.
19. Moulder, J. W. (1985) *Microbiol. Rev.* **49,** 298–337.
20. Jacobs, A. A. C. & de Graaf, F. K. (1985) *FEMS Microbiol. Lett.* **26,** 15–19.
21. Maurelli, A. T., Blackmon, B. & Curtiss, R., III (1984) *Infect. Immun.* **43,** 195–201.
22. Miller, V. L., Taylor, R. K. & Mekalanos, J. J. (1987) *Cell* **48,** 271–279.
23. Taylor, R. K., Miller, V. L., Furlong, D. B. & Mekalanos, J. J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2833–2837.
24. Brubaker, R. R. (1967) *J. Infect. Dis.* **117,** 403–417.
25. Straley, S. C. & Bowmer, W. S. (1986) *Infect. Immun.* **51,** 445–454.
26. Hackstadt, T. & Williams, J. C. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3240–3244.
27. Hackstadt, T., Todd, W. J. & Caldwell, H. D. (1985) *J. Bacteriol.* **161,** 25–31.
28. Peeling, R. W., Peeling, J. & Brunham, R. C. (1989) *Infect. Immun.* **57,** 3338–3344.