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Plasmid-Associated Adherence of *Shigella flexneri* in a HeLa Cell Model

TIBOR PÁL† AND THOMAS LARRY HALE*

Department of Enteric Infections, Walter Reed Army Institute of Research, Washington, D.C. 20307

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The initial interaction of *Shigella flexneri* with HeLa cells was studied at 4°C, a temperature that inhibits parasite-directed endocytosis. It was found that invasive strains were 10-fold more adherent to HeLa cells than were isogenic, noninvasive strains which had lost a 140-megadalton plasmid. Adherent strains were also more hydrophobic than were nonadherent strains.

The cell biology of bacterial invasion has been analyzed by using cultured epithelial cells as surrogates for the intestinal enterocytes which are infected by *Shigella* species in vivo. Transmission electron microscopy of the early stages of infection has demonstrated areas of close apposition between the surfaces of the bacterium and the cultured host cell which are suggestive of receptor-ligand binding (3, 6, 7). These morphological studies suggest that localized binding of bacterial ligands to the host cell glycocalyx induces the endocytosis of adherent shigellae by a "zipper mechanism" similar to that observed in phagocytic cells (2). To quantify interactions with HeLa cells that preceed bacterial invasion, we applied organisms to monolayers at a temperature which immobilizes plasma membrane phospholipids and precludes parasite-directed endocytosis.

Shigellae were grown to the mid-logarithmic phase in Penassay broth with shaking at 37°C, washed once, and suspended at a concentration of 2×10^8 CFU/ml in Dulbecco phosphate-buffered saline (D-PBS) at pH 6.5. This suspension was chilled to 4°C, and 2 ml was added to monolayers of HeLa cells in 35-mm plastic culture dishes (GIBCO Laboratories, Grand Island, N.Y.). After incubation for 18 h at 4°C, the dishes were placed on an orbital shaker (Bellco Glass, Inc., Vineland, N.J.) and rotated at 100 rpm for 2 min. The suspended bacteria were aspirated, and 2.5 ml of cold D-PBS was gently added at the side of each culture dish. The dishes were again rotated on the orbital shaker for 2 min, D-PBS was aspirated, and the washing process was repeated.

Adherence was quantified as CFU per plate by the following procedure. Trypsin (0.25%, 5 ml; GIBCO) was added to each dish, the monolayers were incubated at 37°C for 10 min, and 1.5 ml of normal saline was added to each culture dish, bringing the total volume to 2.0 ml. The HeLa cells and bacteria were suspended and transferred to test tubes, and the HeLa cells were disrupted by sonication for 3 min in an ultrasonic cleaner (Mettler Electronics Corp., Anaheim, Calif.). After serial dilution in PBS, the bacteria were plated onto Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) plates in triplicate, and the CFU per dish were determined.

In initial experiments, the adherence of virulent *Shigella flexneri* serotype 5 strain M90T was compared with that of noninvasive, isogenic strain M90T-55. The latter strain has lost a 140-megadalton virulence plasmid (12). The data in Fig. 1 show that the association of M90T with 35-mm culture dishes was proportional to the number of HeLa cells seeded. In comparison with M90T-55 cells, approximately 10-fold more M90T cells adhered to confluent monolayers (9×10^5 cells per dish). A nonlocalized pattern of bacterial adherence, with a proclivity for the edges of HeLa cells, was seen in nonfixed monolayers which had been stained by indirect immunofluorescence (data not shown). The association of M90T-55 cells with culture dishes was also increased in the presence of HeLa cells, but this increase was not proportional to the number of tissue culture cells.

The relationship of adherence to other plasmid-associated phenotypes was investigated. Expression of the invasiveness phenotype was assessed at 37°C as previously described (4, 5), and the proportion of HeLa cells infected was quantified by microscopic observation of Giemsa-stained monolayers. Hydrophobic interaction chromatography was carried out in 1 ml-columns of phenyl Sepharose CL-4B (Pharmacia, Inc., Piscataway, N.J.) equilibrated with 10 mM phosphate buffer (pH 7.0) containing 4 M NaCl as previously described (16). The contribution of outer membrane proteins to adherence, invasion, and hydrophobicity was evaluated with cultures suspended in D-PBS (pH 7.2) containing 1 mg of trypsin (T-1005; Sigma Chemical Co., St. Louis, Mo.) per ml, incubated for 1 h at 37°C, and washed twice in D-PBS. The effect of mannose was assessed with bacterial cultures suspended in D-PBS containing 1.0% mannose.

Figure 2 illustrates the relationship among adherence, invasion, and bacterial hydrophobicity. *S. flexneri* M90T grown at 37°C was considered to be the positive control in these experiments, and data obtained with other strains were expressed as a percentage of this control (Fig. 2A). Growth of M90T at 30°C, a nonpermissive temperature for expression of the invasiveness phenotype (11), resulted in levels of adherence and hydrophobicity similar to those of M90T-55 (Fig. 2B and C). Likewise, treatment of M90T with trypsin to remove externalized portions of outer membrane proteins resulted in a loss of the plasmid-associated adherence, invasiveness, and hydrophobicity phenotypes (Fig. 2D). Experiments with an isogenic pair of *S. flexneri* 2a strains again showed that a noninvasive variant which had lost a 140-megadalton plasmid ($M4243A_1$) was less adherent and hydrophobic than was the invasive parent (M4243) (Fig. 2E and F). However, these phenotypes were substantially reconstituted in strain $M4243A_1$(pWR110), a transconjugant of $M4243A_1$ which has acquired the 140-megadalton plasmid of M90T (Fig. 2G) (13). Finally, it should be noted that the

* Corresponding author.
† Present address: Institute of Microbiology, University Medical School, Pécs H-7643, Hungary.

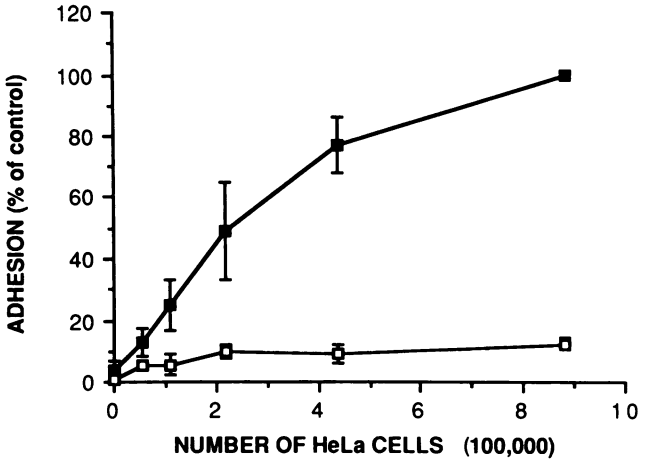

FIG. 1. Relationship between the number of HeLa cells on a 35-mm tissue culture dish and the adherence of *S. flexneri* M90T (■) or M90T-55 (□). The adherence of M90T cells to a 95% confluent monolayer of HeLa cells (9×10^5) was considered to be the maximum potential adherence, and other data, such as adherence in dishes with decreasing numbers of HeLa cells and adherence of M90T-55 cells, are expressed as a percentage of this control, as calculated from triplicate samples in three separate experiments. The bars represent 1 standard deviation.

presence of 1.0% mannose had no effect on adherence or invasion (data not shown).

The data summarized in Fig. 2 indicate that adherence is closely associated with HeLa cell invasion and with bacterial hydrophobicity in *S. flexneri*. The data also indicate that these characteristics are mediated by plasmid-encoded outer membrane proteins. Hydrophobic properties of invasive shigellae have previously been detected by hydrophobic interaction chromatography (15) and Congo red binding (1, 10), and these properties were probably responsible for the 10-fold greater adherence of M90T than of M90T-55 when these strains were added to tissue culture dishes without HeLa cells (data not shown). Nonetheless, hydrophobic interactions alone are clearly not sufficient for the induction of endocytosis by mammalian cells (14), and Fig. 1 indicates that M90T adheres to HeLa cells more readily than to the hydrophobic polystyrene surface of tissue culture dishes. It should also be noted that the retention of invasive *S. flexneri* strains such as M90T and M4243 on phenyl Sepharose CL-4B columns was only 50% that of enteroadherent *Escherichia coli* RDEC1 (data not shown). This observation suggests that invasive shigellae are relatively nonhydrophobic as compared with organisms which colonize the surface of the intestinal epithelium.

It is possible that the relationship between adherence and invasion in *S. flexneri* is analogous to that previously observed for *Yersinia pseudotuberculosis* (8, 9). Cloning of the *inv* chromosomal locus from the latter species into *E. coli* HB101 allows the expression of a specialized outer membrane protein called invasin, and HB101 clones expressing this protein adhere to HEp-2 cells at 4°C and invade them at 37°C. The current data indicate that the adherence of shigellae is also associated with invasion, and the adherence assay described in this communication may prove useful in the identification of plasmid-encoded proteins which are determinants of the invasiveness phenotype in *Shigella* species.

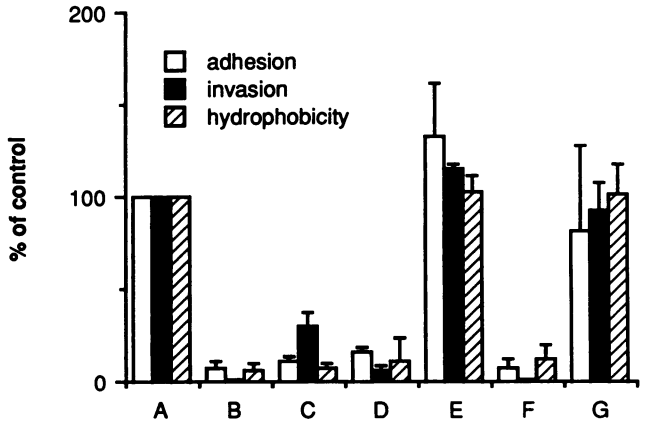

FIG. 2. Relationship between HeLa cell invasion, adherence, and hydrophobicity (determined by hydrophobic interaction chromatography) in *S. flexneri*. Strain M90T grown at 37°C (histogram A) was the positive control for each parameter, and other data are expressed as the mean percentage of this control, as calculated from triplicate samples in three separate experiments. Histograms B (M90T-55), E (M4243), F (M4243A_1), and G [M4243A_1(pWR110)] compare other *S. flexneri* stains to the M90T control. Histograms C (M90T grown at 30°C) and D (M90T exposed to 1% trypsin for 1 h at 37°C) show the effects of experimental manipulation of the control strain. The bars represent 1 standard deviation.

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