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Effect of Guinea Pig or Monkey Colonic Mucus on *Shigella* Aggregation and Invasion of HeLa Cells by *Shigella flexneri* 1b and 2a

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The effects of guinea pig and rhesus monkey colonic mucus preparations on *Shigella* aggregation and invasion of HeLa cell monolayers by *Shigella flexneri* serotype 1b, 2a, and 5 strains were investigated. Guinea pig mucus caused agglutination of *S. flexneri* serotype 1b but not of *S. flexneri* serotype 2a or 5. Guinea pig mucus also inhibited HeLa cell invasion by *S. flexneri* serotypes 1b and 2a. Monkey mucus neither agglutinated any *Shigella* strain nor inhibited HeLa cell invasion.

Invasion of intestinal epithelium by virulent shigellae is a complex process, which depends on both host factors and bacterial factors (8, 10) and occurs naturally only in humans and subhuman primates (16, 17). Adherence to the intestinal mucosa, which is an important early stage for colonization and virulence expression of many microorganisms (15), occurs when various animal species, such as guinea pigs and mice, are exposed to *Shigella* strains under laboratory conditions (1, 7, 11).

Adherence of *Shigella flexneri* to guinea pig colonic mucosal surfaces is calcium, pH, and temperature dependent and is mediated by a carbohydrate-binding substance on the colonic epithelial surface. This adhesin is absent at birth, but it is secreted at adult levels by about 2 weeks of age (1). Preliminary results suggest that the molecular weight of the adhesin subunits is less than 60,000 (D. Mirelman, Y. Nuchamowitz, and M. Izhar, Abstr. J. Cell. Biochem. **15**(Suppl. 7A):9, 1983), and, since the adhesin can be partially washed off the mucosa, it appears to be one of the components of the colonic mucus that coats the epithelial cells. In addition, Duguid and Gillies have found that guinea pig mucus agglutinates *S. flexneri* (3), and more recently Ashkenazi and Mirelman have observed that isolated guinea pig colonic epithelial cells secrete an agglutinin into the surrounding medium within 1 h of isolation (1). Presumably, this solubilized agglutinin, which aggregates *S. flexneri* serotype 1b and some enteroinvasive *Escherichia coli* strains, is related to the mucosal adhesin.

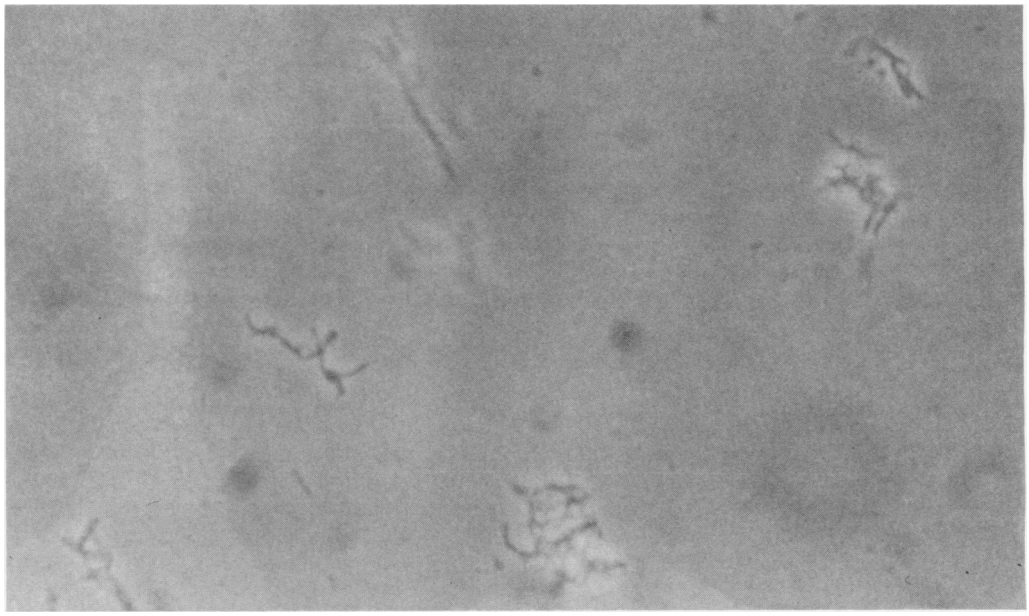

FIG. 1. Agglutination of *S. flexneri* serotype 1b strain M25-8 after 1 h of incubation with a guinea pig colonic mucus preparation.

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TABLE 1. Inhibition of HeLa cell invasion by preincubation of bacteria with different dilutions of guinea pig colonic mucus preparations[a]

Dilution of mucus	% Cells invaded by *S. flexneri* preincubated with guinea pig mucus	
	Serotype 1b strain M25-8 (n = 9)	Serotype 2a strain 2457T (n = 7)
Undiluted	31.4 ± 6.9[b]	36.4 ± 5.0[b]
1:2	37.5 ± 5.8[b]	51.3 ± 3.4[b]
1:4	73.2 ± 4.2[c]	41.6 ± 3.0[d]
1:8	75.1 ± 6.6	40.1 ± 3.1[d]
1:16	79.1 ± 4.3	48.7 ± 4.7[b]
Controls	100	83.6 ± 2.8

[a] When we preincubated *S. flexneri* serotype 1b strain M25-8 or serotype 2a strain 2457T with different dilutions of monkey colonic mucus, 100% of the HeLa cells were invaded. *S. flexneri* serotype 1b was agglutinated by the mucus preparations, while serotype 2a was not. Results are expressed as means ± standard errors of the mean.
[b] $P < 0.01$ compared with controls.
[c] $P < 0.05$ compared with controls.
[d] $P < 0.001$ compared with controls.

Although the physiologic role of this adhesin is unclear, it has been suggested that its presence in the colonic mucus causes bacteria to agglutinate in the mucus layer and facilitates the removal of the bacteria from the intestinal tract by peristalsis (1). This host defense mechanism might be suppressed by opiates and starvation, treatments which greatly enhance the susceptibility of guinea pigs to *Shigella* infections (4, 17). Therefore, it is possible that normal gut motility and mucus secretion protect the guinea pig mucosa and that the mucus agglutinin is involved in this protection. Since rhesus monkeys are much more susceptible to *Shigella* infections than guinea pigs, we compared the abilities of mucus preparations from these two species to aggregate various *S. flexneri* serotypes and to inhibit the invasion of HeLa cells by these organisms.

Crude mucus was isolated from the colons of 300- to 400-g Hartley guinea pigs (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) by using the method described by Laux et al. (12). Briefly, the colon distal to the hepatic flexure was removed, the mucosal layer with the overlying mucus gel was scraped with a scalpel, placed in 4 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-Hanks buffer, and centrifuged at 30,000 × *g* for 25 min, and the supernatant was collected. Protein levels were determined (13), and the protein concentration was adjusted to 2.5 mg/ml. Colons from 1- to 2-year-old rhesus monkeys (sacrificed by penthotal overdose as part of a polio vaccine efficacy evaluation protocol, in which the central nervous systems of monkeys vaccinated 3 to 4 months earlier were examined for evidence of disease) were processed in an identical manner, except that the protein concentration was adjusted to 5 mg/ml. All mucus preparations were frozen at −20°C until they were used.

Samples (100 μl) of twofold serial dilutions of crude mucus prepared in basal medium Eagle (GIBCO Laboratories, Grand Island, N.Y.) were mixed in 96-well plates with 100-μl portions of logarithmic-phase bacterial cultures grown in Penassay broth (Difco Laboratories, Detroit, Mich.). The

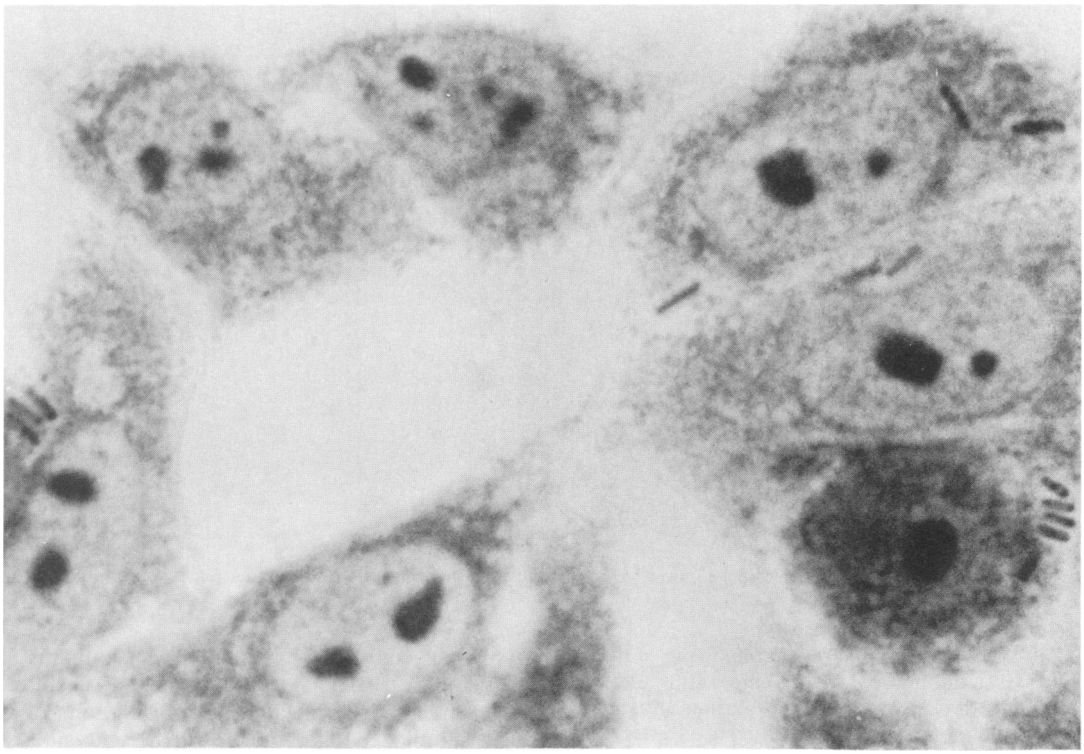

FIG. 2. Inhibition of HeLa cell invasion by *S. flexneri* serotype 1b strain M25-8 after prior incubation with guinea pig colonic mucus. Only a few cells were invaded. Magnification, ×1200.

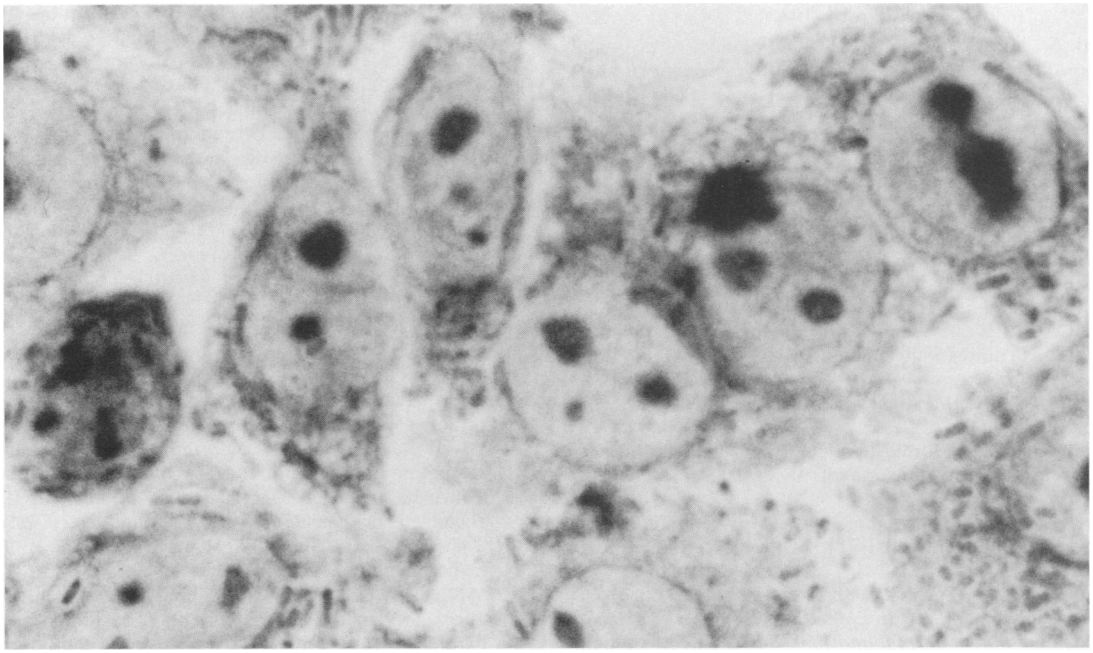

FIG. 3. Invasion of HeLa cells by *S. flexneri* serotype 1b strain M25-8 after prior incubation with monkey colonic mucus. Virtually all of the cells were invaded. Magnification, ×1200.

plates were incubated for 1 h at 37°C in a shaking incubator. Samples (10 μl) were then put on a microscope glass slide and examined under a phase-contrast microscope for the presence of bacterial aggregates. Guinea pig mucus preparations agglutinated *S. flexneri* serotype 1b (strains 1Z and M25-8) but not serotype 2a strain 2457T or serotype 5 strain M90T (14) (Fig. 1). The agglutination process depended on the protein concentration of the preparation, becoming undetectable at a dilution of 1:8 to 1:16. In contrast, monkey mucus agglutinated none of the bacterial strains tested (data not shown).

Logarithmic-phase cultures of *S. flexneri* serotype 1b strain M25-8 or *S. flexneri* serotype 2a strain 2457T adjusted to a concentration of 2×10^8 CFU/ml were used to infect nonconfluent HeLa cell monolayers by using the following modification of our previously described method (9). Samples (1 ml) of a bacterial suspension were mixed with 1-ml portions of twofold serial dilutions of mucus in basal medium Eagle, and the preparations were incubated in a shaking incubator at 37°C for 30 min. Control bacterial samples were treated identically, except that they were mixed with 1 ml of HEPES-Hanks buffer or basal medium Eagle. HeLa cell monolayers were overlaid with 2 ml of a bacterium-mucus mixture or a control suspension, centrifuged, incubated for 2 h at 37°C in 5% CO_2, fixed with methanol, and stained with Giemsa stain. The percentage of infected cells was determined by a microscopic analysis of stained monolayers. A statistical evaluation was performed by using Student's *t* test.

Prior incubation with guinea pig mucus significantly inhibited the invasion of HeLa cells by *S. flexneri* strains whether these strains were agglutinated by the mucus or not (Table 1 and Fig. 2). In contrast, the mucus from monkey colons had no effect on the invasion of HeLa cells despite the fact that the initial protein concentration was twice as high as that of the preparations from guinea pigs (Table 1 and Fig. 3).

Gastrointestinal mucus is not a single, well-defined entity. This term is used to describe the viscous fluid of the intestinal lumen, which includes, in addition to large glycoproteins secreted from epithelial goblet cells, virtually all other constituents normally found in intestinal lumina (6). The mucus preparations used in this study were crude preparations; however, mucus obtained from mice by using a similar procedure contained high-molecular-weight mucus glycoproteins (2, 12). Since *S. flexneri* serotype 1b was agglutinated by our guinea pig mucus preparations, these preparations apparently contained the previously described *Shigella* agglutinin (1). In contrast, no agglutinating activity was detected in colonic mucus preparations obtained from monkeys by using an identical procedure. This suggests that any protective effect accompanying aggregation of shigellae in the mucus layer would not be observed in primates. In addition, the limited number of *S. flexneri* serotypes which are agglutinated by guinea pig mucus logically suggests that this is a minor factor in the protection of rodents against *Shigella* infections.

The fact that guinea pig mucus inhibits the invasion of epithelial cells in vitro in the absence of agglutination is a new observation. The mechanism of this inhibition has not been determined. The mucus may act by providing binding sites which compete with cellular receptors for bacterial surface components. Salivary mucin seems to interfere with the adherence of streptococci to buccal epithelial cells by such a competitive mechanism (18). In addition, guinea pig mucus may physically interfere with the invasion process by forming a viscous barrier which envelopes the bacteria and does not allow them access to epithelial cell binding sites (5). If true, this mechanism should also apply to monkey mucus, which however did not inhibit invasion. Monkey colonic mucus (and by inference, human mucus) appears to lack agglutinating and invasion-inhibiting properties.

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