Production of Mucinase and Neuraminidase and Binding of Shigella to Intestinal Mucin

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ABSTRACT

Production of mucinase and neuraminidase by Shigella spp. and their ability to bind to mucin was investigated. All four species of Shigella produced these enzymes. Virulent and avirulent pairs of Shigella did not differ in their ability to produce the enzymes after 18 h of growth. However, a significant difference in neuraminidase production was noted in Shigella dysenteriae type 1 and S. boydii (12–15) at 10 h growth. Avirulent strains of S. dysenteriae type 1, S. flexneri 2a, and S. boydii bound significantly more amounts of mucin than their virulent counterparts.

Key words: Shigellosis; Diarrhoea; Enzymes of Shigellae.

INTRODUCTION

The mucous layer covering the intestinal epithelium consists of a heterogeneous mixture of proteins and a very large molecular weight glycoprotein called mucin (>2 x 10⁶ dalton) (1,2). This mucous layer represents an important defense mechanism against infection in the gastrointestinal tract (1). Enteropathogens must penetrate the mucous layer to reach the underlying epithelial cells where they can adhere and invade to cause disease (3).

Three extracellular enzymes of Vibrio cholerae are involved in the interaction of the pathogen with intestinal mucus layer. These are mucinase, proteinase, and neuraminidase, collectively described as mucinase complex (4,5). In addition to toxin and other virulence factors, these enzymes are involved in the pathogenesis of intestinal fluid production mediated by V. cholera (4,6). Formal et al. (7) reported that certain strains of Shigella spp. produced an extracellular mucinase which was capable of degrading ovomucin. However, no information is available on the characteristics of the enzyme or its role in shigellosis. In this study we report the production of mucinase and neuraminidase by certain strains of Shigella spp. and their ability to bind to intestinal mucin.

Bacterial strains. Five strains of each of four species of Shigella, S. dysenteriae type 1, S. flexneri 2a, S. boydii (12–15) and S. sonnei, were included in this study (Table). The virulent strains were isolated from patients at the treatment centre of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) in Dhaka, Bangladesh. The avirulent strains (Table) were derived from the virulent ones by repeated subculturing on MacConkey agar (Difco) as described previously (8). The loss of virulence was assessed by the Congo–red binding assay, Sereny test, and HeLa cell invasion assay as described below. Non–pathogenic E. coli strain 36000 was obtained from the Centers for Disease Control (Atlanta, GA, USA). Vibrio cholerae 569B was used as the positive control strain. All the strains were stored at -70°C in Trypticase Soy Broth (TSB, Gibco) with yeast extract (Gibco) 0.6% containing glycerine 15%.

Virulence characteristics of the strains. The Congo–red dye binding capacity of the strains was determined by streaking the strains on trypticase soy
agar (TSA) with yeast extract (Gibco) 0.6% containing Congo red (Fisher Scientific Co., NJ, USA) 0.003%. Plates were incubated at 37°C for 18 h to differentiate between pigmented (Per+) and non-pigmented (Per-) colonies as described previously (9). For the Sereny test, 20 μl of a suspension of the strains in normal saline containing 5 x 10^8 cfu/ml was inoculated into the eye of adult guinea pigs. Strains producing keratoconjunctivitis within 72 h were recorded to be virulent (10). The strains, adjusted to 2 x 10^8 cfu/ml, were cultured for 18 h in broth to infect semi-confluent monolayers of HeLa cells to determine invasiveness (11).

Analysis of plasmid DNA. Plasmids were isolated by a modification of the method of Birnboim and Doly (12) and separated by electrophoresis for 2 h at 30 mA in agarose 0.7%. Preparations of plasmid DNA from corresponding avirulent strains were electrophoresed for 4 h at 30 mA in agarose (0.7%) to detect the deletion in the large plasmid (8). Reference marker plasmids of pBR322, pBR325 (140, 105, 2.7 and 2.0 Mda), R1 (62 Mda), RP4 (36 Mda), and Sa (23 Mda) were used to determine the molecular weight of the plasmids.

Assay of enzyme activities. Strains were grown in 30 ml of TSB for 18 h at 37°C with shaking (250 rpm). The cells were harvested at 10,000 X g for 15 min and the supernatant was filtered (0.45 μm, Millipore), dialysed against phosphate-buffered saline (PBS pH 7.4) overnight at 4°C with constant stirring, and used for enzyme assays. The culture filtrate in serial two-fold dilution was assayed for mucinase activity by the method of Kasuma and Craig (13). Mucin (600 μg protein/ml) was added to each tube and the volume was made up to 0.1 ml with PBS. After 1 h of incubation at 37°C, 50 μl of protease amphotericin sulfate (1.0 mg/ml) was added. The resulting turbidity was assayed after 5 min by measuring the absorbance at 492 nm. The number of units of mucinase in a sample was described as the reciprocal of the highest dilution of filtrate which prevented fibrin clot formation. Neuraminidase was assayed by the quantitation of N-acetyl neuraminic acid released from bovine submaxillary mucin (Sigma, St. Louis, MO) by reacting it with thiobarbituric acid (14). Enzyme activities of outer membrane proteins (15) and cell pellets were tested. Enzyme activity was measured in the periplasmic content of the strains after treating the cell pellets with polymixin B. Each experiment was conducted three times separately. Statistical analysis was carried out using the student's t test.

Isolation and purification of mucin. Mucin glycoprotein was prepared from guinea pig nose and rabbits as previously described (16). Briefly, the small bowel was removed from animals, which were fasted overnight, and placed immediately on ice. The mucosal surface was scraped with a scalpel and the scrapings were pooled in an equal volume of iced, sterile PBS. The mucus preparation was vortexed for 15 min in an ice bath, and was centrifuged at 15,000 X g for 25 min at 4°C. The supernatant was centrifuged and dialysed against PBS for 24 h at 4°C. The dialysate was lyophilised and stored at -70°C as crude mucin. Crude mucin (15-20 mg) was fractionated on a Sepharose 4B column (36 x 2.2 cm; Pharmacia LKB Products, Sweden) with 0.01 M Tris buffer (pH 8.0) and the protein of 2 x 106 mol wt region was dialysed, lyophilised, and treated as pure mucin (16). Human mucin obtained from a patient at post-mortem was also treated as described above. Protein content was estimated by the method of Bradford (17).

Mucin binding assay. The mucin-binding assay was performed as previously described (16). Mucin was resuspended 1:1 (wt/vol) in 0.2 M phosphate buffer (pH 7.4), and iodinated with 125I by the method of Markwell (18), using iodohept (Pierce Chemical, Rockford, Illinois). Specific activity was determined after precipitation in 10% trichloroacetic acid and was expressed as gamma counts per mg of protein. For the assay, Shigella strains were grown overnight at 37°C, with shaking in tryptic soy broth, washed three times in PBS, and resuspended in phosphate buffer (1 x 109 CFU/ml) to a standardised optical density at a wavelength of 600 nm. Labeled mucin (10 μg) was incubated with 1 ml bacteria for 2 h at 27°C. After incubation, samples were centrifuged at 4,000 X g at 4°C for 10 min and resuspended in PBS. The wash was repeated three times with a change in microfuge tubes after the final wash. The final pellet was gamma counted, and the results of the binding assay expressed in μg of mucin glycoprotein adherent to 1 x 109 CFU of organisms.

RESULTS

Plasmid profile and virulence characteristics. The large virulence plasmid (120-140 Mda) was present in all the virulent strains. This virulence plasmid was either absent or largely deleted in size in the avirulent strains. No other alternation in the plasmid profile was noted. Strains possessing the intact virulence plasmid bound the dye Congo-red and produced typical red colonies on Congo-red agar plates. Strains with no virulence plasmid or harbouring partially deleted virulence plasmid produced white colonies. The strains having the large plasmid and which bound the dye were invasive in the HeLa cell assay and induced positive response in the Sereny test. The plasmidless avirulent strains were negative in the HeLa cell assay and failed to cause keratoconjunctivitis in guinea pig eye.

Mucinase and neuraminidase production. All four species of Shigella produced extracellular mucinase and neuraminidase (Table). Enzyme activity in periplasmic extracts was similar to the exhausted enzyme activities. None of these enzyme activities...
were detected in OMPs or cell pellets. The highest mucinase activity was recorded with guinea pig intestinal mucin. This was followed by rabbit and human intestinal mucin and bovine submaxillary mucin (data not shown).

Table. Mucinase and neuraminidase activity in Shigella spp.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Mucinase (U/ml)</th>
<th>Neuraminidase (µg of NANA released/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/- SD 10 h</td>
<td>+/- SD 24 h</td>
</tr>
<tr>
<td>S. flexneri 2a</td>
<td>703 0.033</td>
<td>24 0.02</td>
</tr>
<tr>
<td>VIR</td>
<td>335 0.043</td>
<td>14 0.03</td>
</tr>
<tr>
<td>AVIR</td>
<td>237 0.053</td>
<td>10 0.03</td>
</tr>
<tr>
<td>S. dysenteriae type 1</td>
<td>245 0.057</td>
<td>0 0.03</td>
</tr>
<tr>
<td>VIR</td>
<td>215 0.032</td>
<td>14 0.04</td>
</tr>
<tr>
<td>AVIR</td>
<td>91 0.062</td>
<td>29 0.035</td>
</tr>
<tr>
<td>S. sonnei</td>
<td>409 0.087</td>
<td>14 0.02</td>
</tr>
<tr>
<td>VIR</td>
<td>368 0.077</td>
<td>0 0.02</td>
</tr>
<tr>
<td>E. coli 3600</td>
<td>0 0.00</td>
<td>0 0.00</td>
</tr>
<tr>
<td>V. cholerae 569B</td>
<td>750 0.60</td>
<td>60 0.60</td>
</tr>
</tbody>
</table>

**Discussion**

All the strains investigated in this study produced mucinase and neuraminidase which were quantitatively similar to those produced by *V. cholerae* (1,6,8). No definite association was observed between different species of shigelae regarding production of these enzymes.

Neuraminidase became detectable in supernatant at 4 to 6 h of growth; the enzyme content gradually increased with time. The significant difference in enzyme production between virulent and avirulent pairs of *S. dysenteriae* type 1 and *S. boydii* (12–15) was noted after 10 h of growth. However, the difference in quantitative production of this enzyme after 18 h of growth by virulent and avirulent pairs was not statistically significant. Statistical analysis demonstrated that mucinase and neuraminidase production by virulent strains at 18 h growth were not significantly greater than those produced by avirulent, smooth strains.

**Mucin binding.** The binding of virulent and avirulent shigelae to guinea pig intestinal mucin is shown in the figure. An interesting observation was that avirulent, noninvasive mutants of *S. dysenteriae* type 1, *S. flexneri* 2a, and *S. boydii* (12–15) bound considerably higher amounts of mucin than did their virulent counterparts. There was no significant difference in the binding of virulent and avirulent strains of *S. sonnei* to mucin.

Fig. Microgram of mucin (mean) from guinea pig bound to 1.0 x 10⁹ CFU of various virulent (VIR), and avirulent (AVIR) strains of *Shigella* spp. Avirulent strains of *S. flexneri* 2a, *S. dysenteriae* type 1 and *S. boydii* (12–15) bound significantly more mucin (P=0.006, P=0.0001 and P=0.01, respectively) than their virulent parent strains. Virulent and avirulent pairs of *S. sonnei* did not show any significant difference in their binding abilities. S.FLEX = *S. flexneri*, S.BOYD = *S. boydii*, S.SONN = S. sonnei, S.DYST 1 = *S. dysenteriae* type 1. The results given are mean of three separate assays. The standard deviations are indicated at the top of each bar.

A recent study compared the production of mucinase by one virulent and two avirulent strains of *S. flexneri* 2a (19). It was found that virulent strains produced high levels of mucinase, and production of the enzyme by avirulent strains was not significant. We systematically analysed the production of mucinase by 5 strains of each species of *Shigella* and their laboratory-derived corresponding avirulent strains. We observed that the level of mucinase and neuraminidase production at 18 h growth was higher.
in virulent strains than in avirulent strains but the difference was not statistically significant, which correlates with the results observed by Pirzont et al. (19). This observation indicates that these enzymes do not play a primary role in pathogenesis of shigellosis, but might constitute virulence factors of secondary importance.

Neuraminidase production by *V. cholerae* has been shown to be maximum at 6 h of growth (6). In this study, maximum activity of this enzyme was noted at 18 h of growth, but the enzyme activity of the virulent strains of both *S. dysenteriae* type 1 and *S. boydii* (12-15) was significantly more than their avirulent counterparts at 10 h of growth. Neuraminidase has been found to split sialic acid residues from oligosaccharides to release the GM₁ ganglioside which binds cholera toxin (20,21). This receptor is not required for binding of Shiga toxin, but it does not rule out the possibility that other receptor sites necessary for adherence and invasion are made accessible by this enzyme.

The results of the present study reveal that *Shigella* spp. can specifically bind guinea pig intestinal mucin. The higher amount of enzymes produced by virulent bacteria (Table) may enable them to lyse the mucus barrier to reach the underlying enterocytes, thus permitting only transient association of the pathogen with mucin. On the other hand, avirulent mutants, which produce lesser amounts of mucinase and exhibit stronger binding with mucin, may become entrapped by a mucus layer which may facilitate their expulsion by peristaltic movement. It may also be speculated that virulent bacteria must be efficient in rapidly crossing the mucus barrier to invade the enterocyte; firm and permanent association with mucin may not be considered as a virulence trait.

The present study shows that *Shigella* spp. do produce neuraminidase and mucinase. A more detailed study with purified enzymes and enzyme-deficient mutants needs to be carried out to determine their role, if any, in the pathogenesis of shigellosis.

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