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Degradation of Intestinal Glycoproteins by Pathogenic *Shigella flexneri*

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Intestinal mucin glycoproteins were examined for their ability to sustain growth of pathogenic shigella. Inoculation of germfree cecal mucin glycoproteins with *Shigella flexneri* 4b resulted at 48 h in a 940-fold increase in the enteropathogen concentration. Investigation in vitro of enzymatic degradation by the pathogen led to the identification of a blood group B-degrading glycosidase produced by the bacteria. In in vivo experiments, fecal supernatants of mice monocontaminated with *S. flexneri* 4b contained an α-galactosidase active against the *p*-nitrophenyl-glycoside. This fecal α-galactosidase peaked 5 days after shigella contamination, showing 2.8 ± 1.4 mU of enzyme activity per mg of protein. Contaminated fecal supernatants similarly destroyed the blood group B reactivity of cecal mucin glycoproteins. These data suggested that *S. flexneri* 4b could proliferate within ileocolonic environment by enzymatically degrading mucin glycoprotein sugars.

Studies performed by Hoskins with feces of conventional rats and normal humans have clearly shown that some of the fecal microflora remove sugars from hog gastric mucin by action of blood group-degrading enzymes (8–10). We have similarly identified glycosidases of bacterial origin in cecal contents of conventional animals and bacterially contaminated contents from surgically created jejunal self-filling blind loops (19, 20). These studies suggested that bacterial glycosidases allow fecal or colonic bacteria tc obtain nutrient sugars from intestinal glycoproteins.

Enteropathogens are occasional residents of the intestinal tract. Among them, pathogenic shigella have been known to proliferate in distal intestinal regions (5). In the early stages of shigellosis, numerous bacilli may be found in the lumen of the small bowel (11), but the overt manifestations of bacillary dysentery are associated with proliferation of the pathogen within the ileocolonic environment (7). Little is known about the mechanisms whereby shigella proliferate in the intestine. In experimentally induced bacillary dysentery, histological and immunoflorescent studies reveal niches of shigella closely associated with the coating mucus and membrane surface of colonic cells (23). It is possible that intestinal glycoproteins could provide appropriate carbohydrate substances for survival and growth of shigella if these organisms produce glycosidases.

It is also possible that bacterial glycosidases may play a role in the shigella penetration or adhesion to intestinal surface glycoproteins. In vitro inhibition studies using D-mannose, α-methylmannoside, or yeast mannan appear to suggest that for some shigella strains, the receptor of the intestinal surface is a mannose residue (4, 18). Conceivably, glycosidases from shigella or normal microbiota could uncover receptor sites from the glycoprotein sugars, thus facilitating the invasive process. This report deals with glycosidases identified in a pathogenic species of *Shigella flexneri* and their ability to degrade intestinal glycoproteins.

MATERIALS AND METHODS

Germfree mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) 3 to 4 weeks old were kept in a plastic aseptic isolator (G-F Supply Div., Palatine, Ill.) and given sterile lab chow and water ad libitum. Aerobic and anaerobic cultures of feces were routinely performed to assess sterility. For shigella monocontamination experiments, germfree mice were caged individually in a UV laminar flow hood. An invasive strain of *S. flexneri* 4b isolated from the feces of a patient who developed symptoms of dysentery was kept on slants of antibiotic medium no. 2 (Difco Laboratories, Detroit, Mich.). Contamination was achieved by administering the organism in the drinking water (5×10^7 colony-forming units [CFU]/ml). After contamination, feces were collected daily from each mouse for 2 weeks. For enumeration of *S. flexneri*, fresh feces from each day's collection were weighed, suspended in sterile 0.9% NaCl, homogenized, serially diluted, and plated quantitatively on salmonella-shigella agar (Difco) plates. After 24 h of incubation at 37°C, colonies were counted under a magnifying lens, and the organism was identified by Gram stain, API system (Analytabs Products, Plain View, N.Y.), and

agglutination with antisera. Results were expressed as number of shigella ($\log_{10}$) per gram of feces.

Glycosidase assays. All procedures were carried out at 0 to 4°C. Feces from each day's collection were weighed, suspended in 12 volumes of 0.9% NaCl, homogenized, and centrifuged at 500 × *g* to remove debris. Supernatants were recentrifuged for 30 min at 25,000 × *g* to sediment the bacteria. Final supernatants were concentrated to four times the original stool volumes and assayed for glycosidase activity, using α- and β-*p*-nitrophenylglycosides (Sigma Chemical Co., St. Louis, Mo.). The released *p*-nitrophenol was measured at 420 nm (2). A unit of enzyme is defined as 1 μmol of *p*-nitrophenol per min at 30°C. Test runs in our laboratory at the physiological temperature of 37°C have shown no significant alteration in enzyme activity. Protein content of samples was determined by the method of Lowry et al. (14), using serum bovine albumin as the protein standard.

The degrading ability of shigella-monocontaminated fecal supernatants upon the natural substrate was assessed by the loss of cross-reacting blood group antigen of germfree cecal glycoproteins. Glycosidases were partially purified from fecal supernatants by adding ammonium sulfate to the samples to achieve a 65% saturation of the salt. Samples were mixed intermittently for 5 h and centrifuged at 30,000 × *g*. Pellets containing precipitated glycosidases were suspended in citrate buffer and dialyzed for 2 h against three changes of buffer. Glycoproteins were obtained from ceca of germfree rats (Charles River) by dissolving the contents in distilled water and centrifuging at 9,000 × *g*. Clear supernatants were lyophilized, and the powders were stored. When needed for the enzyme reaction, the lyophilized sample was redissolved to make a 5% (wt/vol) solution in distilled water. Small intestinal mucosa of normal rats was scraped, diluted in 5 mM EDTA, and homogenized, and brush borders were purified according to the method described by Forstner et al. (6). Purified brush borders were suspended in normal saline.

To assay the enzyme's effects on a natural substrate, the partially purified fecal supernatant (100 μl) containing 0.02% sodium azide for bacteriostasis was added to the germfree cecal glycoproteins in a volume reaction of 200 μl and incubated at different intervals. The reaction was terminated by placing the tubes in a boiling water bath for 2 min. After the samples cooled, the loss of blood group reactivity of the germfree glycoprotein was estimated by the hemmagglutination inhibition (HI) reaction. Each incubated sample (0.05 ml) was serially diluted in 0.05 ml of saline. Two hemagglutinating units of antiserum (American Hospital Co., Miami, Fla.) was added followed 2 h later by 0.05 ml of a 1% saline suspension of washed erythrocytes according to the method described by Bendick et al. (3). The highest dilution of antigen cecal glycoprotein that inhibited hemagglutination was defined as the antigen titer. The lectin for the anti-H titer determination was extracted from *Ulex europeus* seeds and purified according to the method of Hoskins and Boulding (10).

In vitro culture system. The production of glycosidase in vitro was determined by inoculating a 5% germfree cecal glycoprotein solution with 7×10^9 CFU of *S. flexneri* 4b. Total volumes of incubation varied between 2 and 5 ml. The mixture was incubated at 30°C in a Dubnoff shaking incubator for different time intervals. At the end of the incubation, equal portions were removed and centrifuged to sediment shigella, and clear supernatants were assayed for glycosidase activity with *p*-nitrophenylglycosides. Glycosidases produced in shigella cultures were also tested against the natural substrate. Glycosidases of the shigella-free supernatants were partially purified by ammonium sulfate precipitation and dialysis and incubated with germfree cecal glycoproteins. At predetermined periods, the reaction was terminated as described before, and the loss of the blood group reactive antigen of the glycoprotein was estimated by HI.

A similar culture system was used to ascertain the growth of shigella in cecal glycoproteins. In this instance, a 5-ml solution of 5% cecal glycoprotein was dialyzed for 24 h against saline and distilled water, respectively, to diffuse out any spontaneously released sugars. The dialyzed cecal glycoprotein was inoculated with shigella to make 10^4 CFU/ml. The mixture was incubated for periods up to 30°C, and equal portions were centrifuged at 20,000 × *g* to sediment the bacteria. Pellets were suspended in saline and cultured for shigella. As a control, normal saline was inoculated with a similar concentration of shigella. Shigella-free supernatants were used to assess the loss of blood group reactivity of the incubated glycoproteins and for glycosidase determinations. A suspension (2 ml) of purified brush borders in saline was similarly incubated with the pathogen to assess its ability to grow in the presence of intestinal membranes. In this instance, an initial 400 × *g* centrifugation sedimented brush borders at the end of the incubation periods. Supernatants were then centrifuged at 20,000 × *g*, and bacterial pellets were suspended and cultured as before.

Disc gel electrophoresis. Partially purified monocontaminated fecal supernatants and shigella cultures (150 to 200 μg of protein) were submitted to discontinuous disc gel electrophoresis in 7.5% acrylamide gels. After a 3-h run at 6.5 mA, gels were stained for protein with Coomassie brilliant blue (16). Destaining of the unbound protein was accomplished with 10% acetic acid. Gels run in parallel were cut in 1-mm slices for identification of enzymatic bands. For determination of the *p*-nitrophenylglycosidase, each slice was incubated in a small volume of pH 5.5 citrate buffer containing the glycoside substrate. After an overnight incubation, the yellow color produced was read at 420 nm. Sliced gels were similarly incubated for 24 h with germfree cecal glycoproteins, and the loss of the blood group reactivity of glycoproteins was estimated by the HI reaction. A decrease of two tubes in the HI test was considered indicative of the presence of a blood group-degrading enzyme in the sliced gel.

Statistics. Comparison between groups was calculated by the nonparametric Mann-Whitney *U* test (22).

RESULTS

Experiments with shigella cultures. The solution of dialyzed germfree cecal glycoproteins inoculated with low numbers of pathogenic *S. flexneri* 4b became turbid after several hours; it revealed a 110-fold increase in the number of shigella after 24 h and a 940-fold increase after 48 h of incubation (Fig. 1A). Conversely, shigella

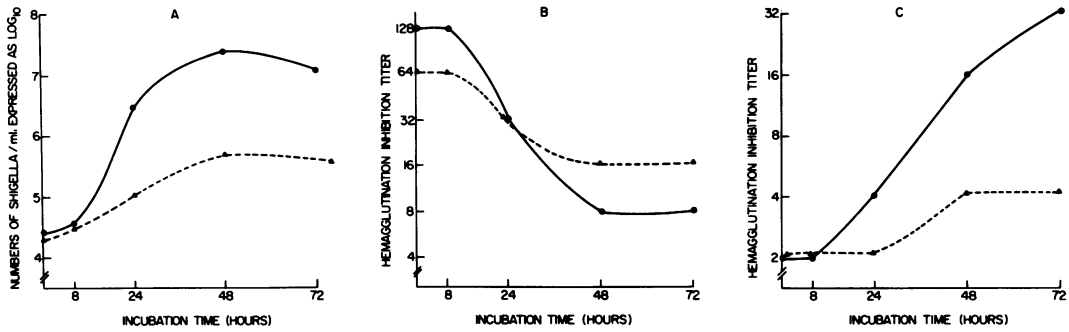

FIG. 1. Cultures of *S. flexneri* 4b with germfree cecal glycoproteins (●) or small intestinal brush borders (▲) as exclusive nutrients. (A) Growth curve; (B) change in blood group B reactivity (numbers on the ordinate represent the dilution of the inhibitory cecal antigen); (C) change in blood group H antigenicity of the underlying antigenic structure.

incubated in saline alone did not exhibit significant growth. In comparison, the growth of shigella was rather slower than that observed in conventional media cultures. This was probably due to the use of a macromolecule glycoprotein containing bound sugars as a simple nutrient source, instead of the highly enriched media with free sugars used in conventional broth. The marked growth of shigella was associated with a significant decrease in the blood group B reactivity of the incubated cecal glycoproteins (Fig. 1B). This loss of the blood group B reactivity was associated with a concomitant increase in the H titer of the underlying H-antigen structure (Fig. 1C). These results with pathogenic shigella are reminiscent of the reported increase in salivary H antigen by action of an α-galactosidase obtained from a fecal strain of *Ruminococcus* (10). Shigella growth was minimal in presence of purified small intestinal brush borders (Fig. 1A). Similarly, there was a negligible decrease in blood group B reactivity (Fig. 1B) and a concomitant slight increase in the H titer of the glycoprotein membrane (Fig. 1C).

In the following experiment, a solution of cecal glycoproteins was inoculated with 7×10^9 CFU of *S. flexneri* 4b and assayed against α- and β-*p*-nitrophenylglycosides for possible production of glycosidases. Before incubation, there was no appreciable activity of any glycosidase. After 24 h of incubation, the culture supernatant revealed a total activity of α-galactosidase, the glycosidase that could destroy the blood group B reactivity of glycoproteins, of 31 ± 18 mU (mean ± standard error). The total activity of the remaining glycosidases was largely low (e.g., β-galactosidase, 8.4 ± 5.5 mU). To examine the action of enzyme on the natural substrate, partially purified culture supernatants were incubated with germfree cecal glycoproteins. The supernatant of the shigella culture decreased by 32-fold (1:64 to 1:2 dilution) the blood group B reactivity of germfree cecal glycoproteins, whereas uninoculated cecal glycoproteins failed to do so.

Experiments in monocontaminated mice. After contamination, feces contained high numbers of *S. flexneri* 4b. Aerobic and anaerobic cultures and identification with the API system failed to reveal any other microorganisms. The shigella concentration reached a maximum at 5 to 7 days postinfection. At that time, the mean ± standard error of the pathogen was 11.1 ± 0.8 ($\log_{10}$) CFU/g of feces (Fig. 2A). Thereafter, the concentration of *S. flexneri* 4b decreased and stabilized in the 2nd week at about 9.5 ($\log_{10}$) CFU/g. These numbers were similar to those obtained by Maier et al. (15) with *S. flexneri* 2a.

Feces of germfree mice contained very low activities of those glycosidases that act on β-linkages and thus do not destroy blood group reactivity. Specific activity of β-*N*-acetylglucosaminidase was 0.40 ± 0.09 mU/mg of protein, and that of β-galactosidase 0.17 ± 0.05 mU/mg of protein. Between days 3 and 5 of contamination, there was a moderate but consistent increase in the specific activity of β-*N*-acetylglucosaminidase to 0.80 ± 0.10 mU/mg of protein and of β-galactosidase to 0.54 ± 0.25 mU/mg of protein. In contrast, contamination of mice with pathogenic shigella resulted in a sharp elevation of the activity of α-galactosidase (Fig. 2B). In the germfree state and against the *p*-nitrophenyl-galactoside, fecal supernatants contained no or negligible enzyme activity. Though evidencing large variation from animal to animal, the α-galactosidase activity on day 5 was significantly higher than before monocontamination. On that day, the enzyme activity was 132 ± 69 mU/ml of feces, 2.8 ± 1.42 mU/ml of protein, and 208 ± 12.3 mU excreted in 24 h. These values were significantly different ($P < 0.02$) from those in the germfree states. This was partly due to the insignificant enzyme activity of germfree feces.

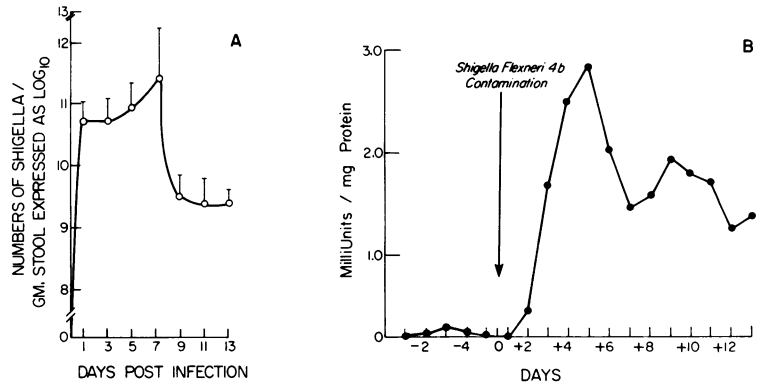

FIG. 2. (A) Curve of *S. flexneri* 4b concentration in feces of seven mice monocontaminated with the pathogen. Each point and bar represents the mean ± standard error of shigella per gram of feces. (B) Mean specific activity of α-galactosidase in fecal supernatants of five mice in the germfree state and after contamination with *S. flexneri* 4b. The appearance of α-galactosidase in fecal supernatants revealed a 20 to 50% ± standard error variation from the mean activity during the 1st week and a 50 to 70% ± standard error variation in the 2nd week after shigella monocontamination. The arrow indicates the beginning of shigella monocontamination. The numbers with negative sign in the abscissa represent days in the germfree state; numbers with positive signs represent days after shigella monocontamination.

On the 5th day of contamination, the average weight of feces was 1.7 g. This weight was similar to the 2.3 g excreted by animals in the germfree state.

Incubation of partially purified monocontaminated fecal supernatants with germfree cecal glycoproteins resulted in the loss of the blood group B reactivity of cecal glycoproteins by 16-fold after 4 h and by 32-fold after 24 h of incubation (Fig. 3).

Separation of shigella α-galactosidases. Disc gel electrophoresis of partially purified shigella cultures and fecal supernatants showed similar protein bands. Likewise, sliced gels incubated with the *p*-nitrophenylglycoside and with cecal glycoproteins revealed a single enzymatic band active against both substrates (Fig. 4A,B). The α-galactosidase of monocontaminated fecal supernatants had an R_f of 0.33 to 0.34. In vitro shigella cultures revealed an α-galactosidase with an R_f of 0.36 to 0.37. The slight difference in R_f was due to variations in the mobility of the reference dye front. The α-galactosidase of the in vivo and in vitro preparations coincided with a single protein band (Fig. 4A and B).

DISCUSSION

In recent years, a number of experimental studies have demonstrated that carbohydrates of gastric or intestinal glycoproteins can be degraded and used for nutrition by normal fecal or colonic-type bacteria (8–10). The present study reveals that colonic glycoproteins can similarly be utilized as nutrients by a pathogenic strain of *S. flexneri*. The marked growth of the pathogen in a medium of cecal glycoproteins would support this view. It is recognized that the growth of shigella requires a hexose as a carbon source (15). The variety of sugars present in the oligosaccharide chains of mucin glycoproteins would offer the pathogen ubiquitous carbon sources. Indeed, our data show that incubation of soluble colonic mucin glycoproteins with pathogenic shigella resulted in loss of the blood group reactivity of the oligosaccharide chains. This loss could have been achieved only by removal of the terminal galactose from the carbohydrate moiety of colonic glycoproteins. The free galactose could then be utilized by the pathogen for metabolism, energy, and growth. Our previous experiments with blind loop animals appear to corroborate this scheme (19). Hence, radioactively labeled oligosaccharide chains of glycoproteins, incubated with colonic bacteria obtained from blind loop sacs, resulted in the production of labeled short-chain fatty acids. Concomitantly, we found a significant decrease in the sugar content and blood group reactivity of intestinal glycoproteins.

In further elucidating the aforementioned mechanism, the present work offers evidence that *S. flexneri* 4b is a producer of glycoprotein-degrading glycosidases, particularly a blood group B α-galactosidase. Electrophoresis of both shigella cultures and monocontaminated fecal supernatants revealed a single α-galactosidase band which split off the *p*-nitrophenylglycoside and degraded the blood group B reactivity of cecal glycoproteins (1, 10). To my knowledge, this is the first detailed study of a blood group-degrading enzyme produced by a pathogenic strain of *Shigella*.

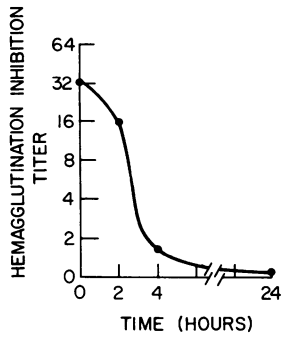

FIG. 3. Loss in blood group B reactivity of germfree cecal glycoproteins after incubation with fecal supernatants of shigella-monocontaminated mice. Numbers on the ordinate represent the dilution of the inhibitory cecal antigen.

The in vitro inoculation of germfree cecal glycoproteins and in vivo monocontamination with *S. flexneri* confirmed the bacterial origin of the α-galactosidase. In the monocontaminated mice, the time after shigella colonization at which α-galactosidase peaked in fecal supernatants correlated closely with the quantity of shigella concentration recovered in feces. In fact, the decrease of the α-galactosidase in the 2nd week was similarly correlated by a lower shigella population. The blood group B-degrading enzyme produced by the pathogenic shigella appeared to act on the oligosaccharide chains as an exogalactosidase. After the loss of the blood group reactivity, the H antigenicity of the cecal glycoproteins increased, presumably by uncovering the fucose residue. It is likely that glycosidase (20) other than the blood group B-degrading enzyme contributed to the removal of sugars from the colonic glycoprotein molecule. This would agree with the increased recovery of nonblood group-degrading β-*N*-acetylglucosaminidase and β-galactosidase in shigella-monocontaminated fecal supernatants. These results also suggest that shigella glycosidases, unlike glycosidases produced by colonic-type bacteria (19), appear to act more readily upon soluble mucin glycoproteins than on particulate small intestinal brush borders. In this regard, it is noteworthy that overt bacillary dysentery is largely confined to the large bowel. It is possible that the pathogenic shigella produce glycosidases which are qualitatively different from those produced by normal enteric bacteria.

My findings of shigella proliferation in the intestinal medium agree with experimental and clinical studies of shigellosis. Experimentally, Labrec et al. (12) found that the population of a virulent strain of shigella in ligated guinea pig ileum increased by 100-fold at 24 h after oral

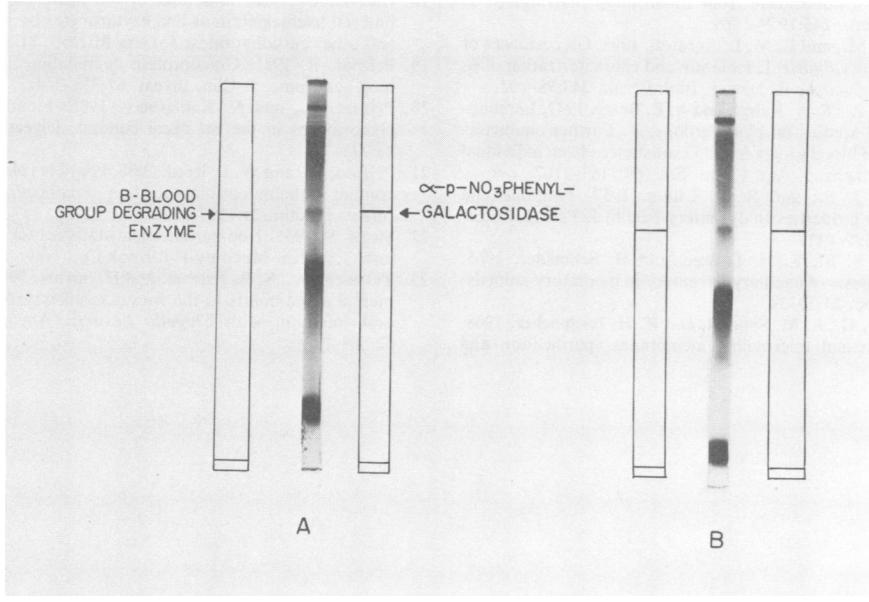

FIG. 4. Disc gel electrophoresis of enzyme preparations. (A) Gels applied with fecal supernatants of shigella-monocontaminated mice. Left gel: Electrophoretic band which destroyed the blood group B reactivity of germfree cecal glycoproteins. Right gel: Electrophoretic band which hydrolyzed the α-*p*-nitrophenylgalactoside. Both enzymatic bands coincided with a protein band in the middle gel. (B) Gels applied with supernatant from shigella culture; left gel shows the blood group B-degrading enzyme band; right gel shows the α-*p*-nitrophenylgalactosidase band; middle gel shows the protein bands.

infection. Clinically, Levine et al. were able to induce diarrheal disease in volunteers by infecting them with doses of 10 to 200 shigella organisms (13). At the height of the infective process, feces contained 10^6 to 10^{10} shiga organisms/g. In addition, these findings are of relevance to the pathogenesis of shigellosis in view of our previous observation (21). In that study, shigella associated in higher numbers with germfree cecal mucosa that had been incubated previously with shigella-monocontaminated or normal fecal supernatants. These contaminated fecal supernatants were shown to contain bacterial glycosidases. It could then be postulated that glycosidases, such as those identified in the present investigation, might not only promote colonization, but even mediate shigella penetration into the intestinal mucosa. Future experiments designed to include identified glycosidases in shigella-cell penetration systems will be of obvious importance to elucidate these postulated pathogenic mechanisms.

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