Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1092–1095, February 1983
Medical Sciences

Vibrio cholerae hemagglutinin/lectin/protease hydrolyzes fibronectin and ovomucin: F. M. Burnet revisited

(lactoferrin/mucinase/enterotoxin/adherence/immunity)

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Communicated by James O. Davis, November 24, 1982

ABSTRACT **Cholera vibrios produce a single polymeric protein that (*i*) causes hemagglutination; (*ii*) appears to participate in their attachment to gut epithelium; (*iii*) may mediate their detachment from gut epithelium; and (*iv*) is a protease that hydrolyzes fibronectin and mucin, cleaves lactoferrin, and nicks the A subunit of the choleragen-related heat-labile enterotoxin of *Escherichia coli*.**

In 1947, F. M. Burnet reported the discovery of a mucinase, elaborated by *Vibrio cholerae*, which participated in the desquamation of epithelium from pieces of guinea pig intestine *in vitro* (1, 2). Even though Burnet meticulously avoided overstating his conclusions, the observations inspired a flurry of activity among cholera researchers, which led to recommendations that cholera mucinase be included in cholera vaccines (3–6). The concept that the cholera mucinase played a significant direct role in the pathogenesis of choleraic diarrhea was, however, short-lived. Gangarosa *et al.* (7) showed that the integrity of the intestinal epithelium was unaltered during the disease in human beings; Gordon (8) found that the disease was not an "exudative enteropathy"; Phillips (9) observed that the intestinal secretions were low in protein and isotonic; and Finkelstein *et al.* (10) found that the enterotoxin (choleragen) which caused the diarrhea of cholera could be separated from the cholera mucinase and that the mucinase was not diarrheagenic. Our current observations, however, indicate that Burnet's mucinase may still be involved in the pathogenesis of cholera even though it is not responsible directly for the diarrhea of cholera.

Recent efforts in our laboratory have been directed toward understanding the mechanism(s) by which the cholera vibrios elude the host intestinal clearance mechanisms of mucus secretion and peristalsis (11). We have isolated (12), from broth cultures of *V. cholerae*, a hemagglutinin (HA), which we previously called cholera lectin (13). This protein, a polymer of 32,000 M_r subunits, which appears to participate in the attachment of cholera vibrios to intestinal epithelium, has inherent protease activity (12). Specific antibody against the purified HA inhibits its protease function and also inhibits attachment of *V. cholerae* to intestinal epithelium (12).

The present report shows that the HA/lectin/protease is produced in both a cell-associated and a soluble form *in vivo* and that it hydrolyzes fibronectin, mucin, and lactoferrin—three proteins that may participate in host defense against cholera. It also nicks the A subunit of the choleragen-related heat-labile enterotoxin (LT) of *Escherichia coli* (14) and thus, by inference, it may also be responsible for the cleavage of the A subunit of the cholera enterotoxin (15) that results in its activation.

METHODS

***V. cholerae* Grown *in Vivo*.** Infant rabbits were inoculated intra-intestinally (16) with three representative virulent strains of *V. cholerae*: CA 401 [from which the soluble HA was isolated (12)], 569B (16), and 3083 [the parent of the Texas Star-SR avirulent A^-B^+ mutant candidate vaccine strain (17, 18)]. The infant rabbits (nine were inoculated with CA 401, three with 569B, and five with 3083) were sacrificed approximately 18 hr later and intestinal fluids, ranging between 6 and 17.5 ml and containing $2.0–42 \times 10^7$ live cholera vibrios per ml, were harvested and lightly centrifuged ($200 \times g$ for 5 min) to sediment the nonbacterial solids. The supernates were subjected to higher speed ($12,000 \times g$ for 20 min) centrifugation to sediment the bacteria. The twice-washed [in KRT buffer (19)] bacterial pellets and the supernates were tested for HA activity on both "responder" and "nonresponder" chicken erythrocytes (RBC) (12, 19).

Purification of HA. The HA/lectin/protease of strain CA 401 was purified to homogeneity by a sequence involving ammonium sulfate precipitation, gel filtration on Sephadex G-75, and isoelectric focusing as described (12).

Protease Activity. Protease activity was initially detected by using a single-diffusion technique in agar gel containing skim milk as a substrate (20). Zones of clearing were roughly proportional to the amount of protease present. Although not precisely quantitative, this was a convenient method for following protease activity during purification. For screening potential protein substrates, the rate of release of radioactive material not precipitable with 10% trichloracetic acid was determined after incubation at 37°C with purified HA and ^{125}I-radioiodinated proteins (21). ^{125}I-Labeled proteins, incubated in buffer in lieu of HA, were included as controls. The following proteins were used: bovine serum albumin (Sigma); two preparations of human plasma fibronectin (one purchased from Calbiochem–Behring and the other a gift from Alex Kurosky); lactoferrin and secretory IgA purified from human milk (22); and rabbit IgG purified according to ref. 23. Cleavage products of noniodinated proteins were detected by $NaDodSO_4$/polyacrylamide slab gel electrophoresis (24) after incubation with purified HA at room temperature overnight at an enzyme-to-substrate ratio of 1:150, in pH 8.0 buffer containing sodium azide. LT from an *E. coli* strain isolated from a human patient with diarrhea (H-LT) purified in unnicked form (14) and purified choleragen (25) were included in this analysis. Protein determinations were done by the method of Lowry (26).

Mucinase activities of the crude $(NH_4)_2SO_4$-precipitated HA, the Sephadex G-75 partially purified material, and the isoelectrically focused pure HA (12) were determined with ovomucin substrate by modifications of the technique of Burnet (1,

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Abbreviations: HA, hemagglutinin; LT, heat-labile enterotoxin; H-LT, LT purified from an *E. coli* strain isolated from a human patient; RBC, erythrocytes.

2) described previously (10). The anti-mucinase activity of rabbit anti-HA (12) was determined by serially diluting the antiserum (0.25 ml), adding an equal volume of Sephadex G-75-purified HA diluted to contain approximately 6 mucinase units, and then adding 0.5 ml of ovomucin diluted to contain 2 clot-forming units (10). Cetyltrimethylammonium bromide (CTAB) was added after 30-min incubation at 37°C.

RESULTS

Production of HA/Protease *in Vivo*. Each of the gut fluid supernates contained HA that, like the crude and purified HA, had activity (titers from 1:8 to 1:64) on responder but not nonresponder chicken RBC. Most significantly, the washed, sedimented bacteria from rabbits infected with CA 401 and 569B [strains that do not usually exhibit cell-associated HA *in vitro* (19)], in each instance, directly agglutinated responder but not nonresponder RBC. Bacterial sediment from two rabbits infected with strain 3083, an El Tor biotype that produces a mannose-sensitive cell-associated HA in addition to the soluble HA/lectin/protease *in vitro* (19), also agglutinated nonresponder RBC, whereas the bacteria from a third 3083-infected rabbit tested agglutinated only responder RBC. Each of the supernatant fluids was also found to have protease specificity similar to purified HA/lectin on bovine serum albumin, fibronectin, and lactoferrin (see below).

Protease Activity. Preliminary screening, using ^{125}I-labeled proteins, revealed that the Sephadex G-75-purified and the pure isoelectrically focused HA behaved identically on the panel of substrates tested. Trichloroacetic acid precipitability of bovine serum albumin and fibronectin was markedly reduced (30–40%) within 1 hr of incubation. Lactoferrin and rabbit IgG were not significantly affected and the trichloroacetic acid precipitability of secretory IgA was only marginally affected (≈10%).

Analysis of the reaction mixtures by NaDodSO$_4$/polyacrylamide slab gel electrophoresis (Fig. 1) revealed, however, that even though there was no apparent reduction of trichloroacetic acid precipitability of the lactoferrin, it yielded two observable fragments (M_r ≈37,000 and ≈55,000) (Fig. 1, lanes 8 and 9). The results were identical when apolactoferrin or iron-saturated lactoferrin were substituted for the native lactoferrin shown in Fig. 1. Fibronectin, which, in our hands as in other laboratories (27), exhibited several protein bands prior to treatment with HA/protease, was significantly digested (Fig. 1, lanes 1 and 2).

Despite overnight incubation with HA/protease, H-LT, whose A subunit was intact prior to treatment, was apparently hydrolyzed only at a single site in the A subunit, yielding (Fig. 1, lanes 10 and 12) a fragment that corresponded to the A_1 peptide of the nicked (activated) form of H-LT that had been isolated from an *E. coli* culture supernate. The fragment also corresponded to the A_1 peptide of cholera enterotoxin that was also already nicked and to the A_1 peptide of H-LT that was formed by limited trypsin treatment of unnicked toxin (results not shown). Already nicked cholera toxin (results not shown) or H-LT (Fig. 1, lanes 13 and 14) were unaffected by treatment with HA/protease.

In accord with the observations with ^{125}I-labeled proteins, rabbit IgG was minimally, if at all, altered (Fig. 1, lanes 6 and 7). Treatment of secretory IgA with HA/protease resulted in the disappearance of a large molecular weight band; however, minimal, if any, changes in the secretory piece and heavy and light chains were observed (Fig. 1, lanes 4 and 5). It is not clear from these results whether a functional alteration has occurred.

The purified HA/protease is functionally identical with the cholera mucinase described earlier by Burnet (1, 2). A mucinase unit was approximately 30 ng of the pure protein. Although precipitability of treated ovomucin with cetyltrimethylammonium bromide was destroyed, the major protein seen in NaDodSO$_4$/polyacrylamide slab gel electrophoresis of the ovomucin preparation (at about M_r 14,000) was not visibly affected. The ovomucinase activity was inhibited by rabbit antiserum against the purified HA at a final dilution of 1:640. This serum, which gives only a single precipitin band with concentrated crude *V. cholerae* culture supernates (12) and inhibits attachment of *V. cholerae* (12), also inhibits the mobility of the protease activity in skim milk agar (Fig. 2). The protease activity is not affected by an irrelevant heterologous antigen–antibody precipitate (in this case, cholera enterotoxin and its antibody).

DISCUSSION

The present results confirm and extend our previous observations that cholera vibrios elaborate, *in vivo* and *in vitro*, a HA

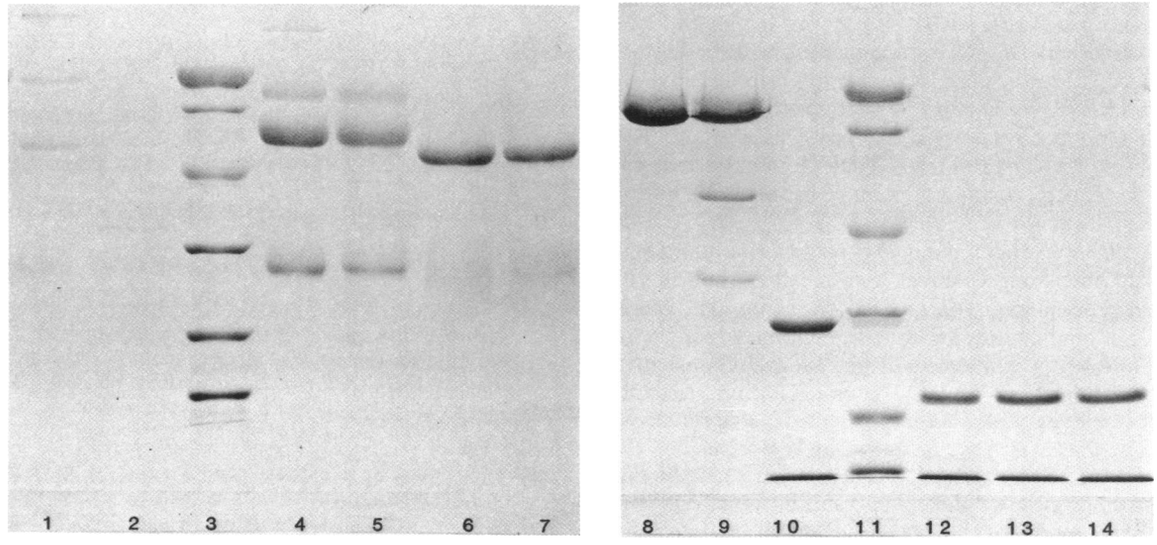

FIG. 1. Coomassie blue-stained NaDodSO$_4$/12% polyacrylamide slab gel electrophoresis. Lane 1, fibronectin, untreated; lane 2, fibronectin, treated with HA/protease; lane 3, molecular weight markers, from the top, 92,500, 66,200, 45,000, 31,000, 21,500, and 14,400; lane 4, secretory IgA, untreated; lane 5, secretory IgA, treated with HA/protease; lane 6, rabbit IgG, untreated; lane 7, rabbit IgG, treated with HA/protease; lane 8, lactoferrin, untreated; lane 9, lactoferrin, treated with HA/protease; lane 10, unnicked H-LT, untreated; lane 11, markers as in lane 3; lane 12, unnicked H-LT, treated with HA/protease; lane 13, nicked H-LT, untreated; lane 14, nicked H-LT, treated with HA/protease.

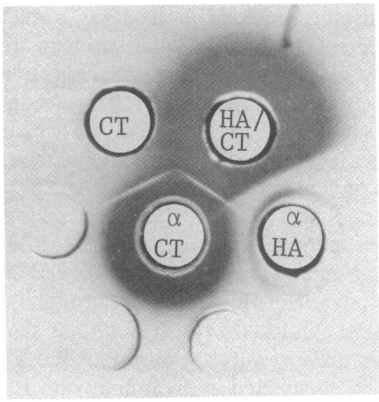

FIG. 2. Inhibition of protease activity of HA/protease by rabbit anti-HA serum in skim milk agar (20). Wells contain cholera toxin (CT), cholera toxin plus HA/protease (HA/CT), anti-HA (α HA), and anti-cholera toxin (α CT). The anti-HA blocked the diffusion of the proteolytic activity of the HA/protease, which was not affected by an irrelevant unrelated antigen–antibody reaction (anti-cholera toxin vs. cholera toxin). The (irrelevant) protease activity of the anti-cholera toxin serum was not affected by the anti-HA.

that has inherent protease activity on several host proteins that are presumed to be significant factors in specific and nonspecific resistance to colonization of the small bowel.

We have apparently rediscovered the cholera mucinase that was recognized by Burnet in 1947 (1, 2). Although subsequent reports eliminated any direct role for the mucinase in the causation of the diarrhea of cholera, mucinase activity could be important in facilitating vibrio penetration of the mucus layer to reach buried epithelial binding sites (11).

Fibronectin is an adhesive high molecular weight glycoprotein that is present on the surface of mammalian cells (28, 29) and that has been shown to be synthesized by the crypt cells of the intestinal epithelium (30). Whereas fibronectin is believed to facilitate the attachment of Gram-positive bacteria to host cells and to participate in opsonization (29, 31), it has been reported to interfere with the adherence of *Pseudomonas aeruginosa* to epithelial cells (32). Our results do not permit a conclusion as to whether fibronectinase is responsible for binding of the vibrios to the mucosal surface and, possibly, their subsequent detachment (11); whether it enables the vibrios to penetrate the fibronectin layer and then attach to another substrate; or whether the fibronectinase activity is interesting but irrelevant. In these regards, it will be important to determine whether more (or less) fibronectin is present on intestinal epithelial cells of young (more susceptible) rabbits (or mice) than on older (more resistant) animals and whether the amount of fibronectin diminishes during *V. cholerae* infection.

Lactoferrin has also been assumed to play a major role in host defense by withholding essential iron and thus reducing its availability to the microbes in mucosal secretions (33). Lactoferrin has two binding sites for iron. The enzymatic activity of the HA/protease appears to be equally effective on the native (partially saturated), apo-, and iron-saturated forms of lactoferrin, and it is not yet clear whether this may be a vibrio mechanism for countering iron sequestration by lactoferrin.

The HA/protease has yet another activity that may be important in pathogenesis of cholera in that it nicks, at a specific site, the A subunit of the choleragen-related LT of *E. coli* and thus may be the mechanism by which cholera enterotoxin is activated.

Possible additional effects on immunoglobulins are not clear-cut. A 10% decrease in trichloroacetic acid-precipitable radioactive material with purified secretory IgA was observed, but this was not accompanied by significant reduction in apparent molecular weight of the component subunits. Thus the enzyme is clearly not the same as the IgA proteases described earlier (34) and it is moot whether there may be some effect on the antigen-binding region of secretory IgA. Observed changes in IgG were even less marked, but traces of additional protein-staining bands appeared after HA/protease treatment (Fig. 1). It is noteworthy that Schneider and Parker (35) alluded to the observation that *V. cholerae* mucinase was also active on immunoglobulin. The same workers reported earlier (36) that *V. cholerae* protease-deficient mutants were generally less virulent in the infant mouse. Their antiserum produced multiple precipitin bands with our crude HA but also precipitated our purified material. Their protease, however, was not available for comparison (C. Parker, personal communication).

It might be argued that it could be disadvantageous to the vibrios to elaborate a soluble HA/protease that was shown (12, 13) to block attachment. It was therefore especially significant to observe that the soluble HA/protease is also produced in a cell-associated form *in vivo*.

It is remarkable that a single protein produced by cholera vibrios can, at least in theory, so contribute to their virulence. Obviously further studies are needed to define its contributions to the pathogenesis and immunology of cholera. It will be of considerable interest to determine (*i*) whether antibodies to the HA/protease can contribute to immunity against cholera; (*ii*) whether convalescents from cholera or recipients of the Texas Star-SR attenuated live vaccine (17), who are immunized against cholera (37–39), develop a secretory (or humoral) immune response against the factor; and (*iii*) whether other pathogenic bacteria, especially, for example, enteropathogenic serotype *E. coli* (EPEC), utilize similar factors to facilitate their attachment to epithelial surfaces.

This study was supported in part by National Institutes of Health Grants AI-16764, AI-16776, and AI-17312.

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