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Neuraminidase Production by *Vibrio cholerae* O1 and Other Diarrheagenic Bacteria

SHAHJAHAN KABIR,*† NAYEEMA AHMAD, AND SHOWKAT ALI

Department of Microbiology, University of Dhaka, and the International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh

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Vibrio cholerae O1 strains belonging to both biotypes (classical and El Tor) and both serotypes (Ogawa and Inaba) produced neuraminidase which was released rather than cell bound. Classical strains made more neuraminidase than did El Tor strains. About one-third of *V. cholerae* non-O1 strains and one-fourth of *Aeromonas hydrophila* strains were neuraminidase positive. Strains of enterotoxigenic *Escherichia coli*, *Vibrio parahaemolyticus*, and *Shigella* spp. did not produce detectable neuraminidase.

Vibrio cholerae is a gram-negative bacterium which colonizes the small intestine and secretes an enterotoxin. It also produces a variety of enzymes, such as neuraminidase, mucinase, collagenase, lecithinase, lipase, and proteinase (15). Although it is well established that cholera toxin is the causative agent of diarrhea (6), the role of these enzymes in the pathogenicity of *V. cholerae* has not been elucidated. There are two biotypes of *V. cholerae*: classical and El Tor. The El Tor strains, in general, produce a higher ratio of mild/asymptomatic to symptomatic infections than do classical strains. The reason for the difference in the epidemiology of El Tor and classical cholera is not well understood. Several pathogenic bacteria produce neuraminidase in addition to recognized toxins, and it has been speculated that neuraminidase may act as a virulence factor (13). *V. cholerae* classical strains are known to produce neuraminidase (1, 3), but a detailed study on the production of neuraminidase by El Tor strains has been lacking in the literature. There are several other enteric bacteria, such as *V. cholerae* non-O1, *Vibrio parahaemolyticus*, *Aeromonas hydrophila*, enterotoxigenic *Escherichia coli*, and *Shigella* spp., which cause diarrheal illness in humans (4). Whether these bacteria produce neuraminidase has not been adequately studied. Therefore, the present study was initiated to investigate in detail the production of neuraminidase by a range of enteric bacteria causing diarrheal illness in humans.

Most of the strains used were clinical isolates obtained from the collection of the International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh. Unless otherwise stated, bacteria were cultured in 3% peptone-water at 37°C with continuous shaking up to the stationary phase of growth, which was monitored by measuring cell turbidity at 600 nm. The culture was harvested by centrifugation at 12,000 × *g* for 15 min at 7°C. The supernatant was filtered through a membrane filter (0.45 μm; Millipore Corp.) and dialyzed against 0.1 M acetate buffer (pH 5.5) containing 0.02 M $CaCl_2$ for a period of 48 h at 4°C. Portions of 0.2 ml of the dialyzed culture supernatant were incubated with 0.1 ml of a solution of bovine submaxillary mucin (10 mg/ml; Sigma Chemical Co., St. Louis, Mo.). The final volume of the reaction mixture was brought to 0.5 ml with 0.1 M sodium acetate buffer (pH 5.5) containing 0.02 M $CaCl_2$. The incubation time for screening culture supernatants was 16 h. The amount of released *N*-acetylneuraminic acid in the assay mixture was determined by the thiobarbituric acid method of Aminoff (2).

V. cholerae O1 strains, regardless of the biotypes and serotypes, produced neuraminidase (Table 1). A statistical analysis done by Student's *t* test demonstrated that neuraminidase production by classical biotypes was actually greater than that by El Tor biotypes ($P < 0.001$). Besides, classical strains produced neuraminidase which had a higher specific activity than did that secreted by El Tor and non-O1 strains ($P < 0.001$; Table 2).

V. cholerae O1 strains were cultivated in peptone-water, as well as in a chemically defined medium containing glutamine, serine, aspartic acid, and arginine. Although both media supported good growth, the production of neuraminidase was significantly reduced in the synthetic medium ($P < 0.02$; Table 3).

To determine the amount of secreted and cell-bound neuraminidase, *V. cholerae* O1 cultures were harvested by centrifugation at 12,000 × *g*. The cell pellet was suspended in phosphate-buffered saline (PBS; 0.01 M [pH 7.4]) to 1/25 of the original volume. The suspension was divided into three parts and treated as follows. (i) polymyxin B (Sigma) was dissolved in PBS (10 mg/ml) and added to the cell suspension to a final concentration of 2 mg/ml. The mixture was shaken at 30°C for 30 min. (ii) The cell suspension in PBS was sonicated in a Braunsonic ultrasonicator 10 times in an ice bath for 1 min each time. (iii) The cell suspension in PBS was shaken at 30°C for 30 min. These suspensions were further centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant from each portion was passed through a Millipore filter (pore diameter, 0.45 μm) and assayed for neuraminidase activity. The release of cell-associated protein toxin for *V. cholerae* (11), *E. coli* (9), and *Shigella dysenteriae* 1 (7) has been shown to occur at an antibiotic concentration of 2 mg/ml, or even at a lower dose.

It may be possible that a *V. cholerae* O1 strain releasing low levels of neuraminidase possesses higher levels of cell-associated neuraminidase. Therefore, we examined *V. cholerae* cultures which were secreting both high and low levels of the enzyme. Neuraminidase appears to be a transported protein, as all *V. cholerae* O1 cultures contained very little cell-bound neuraminidase (Table 4). Enterotoxigenic strains of *E. coli*, after being treated with polymyxin B or being sonicated, were assayed for cell-associated neuraminidase

* Corresponding author.

† Present address: Tobaksspinnargatan 5, 117 36 Stockholm, Sweden.

TABLE 1. Production of neuraminidase by *V. chloerae* O1 strains[a]

Classical strain	Serotype	NANA released (μmol)[b]	El Tor strain	Serotype	NANA released (μmol)[b]
KB 5	Ogawa	1.56	U-10198	Ogawa	0.68
KB 358	Inaba	1.37	18600	Ogawa	0.46
KB 357	Ogawa	1.08	U-2439	Ogawa	0.42
T-19767	Ogawa	0.91	W-13093	Ogawa	0.38
569B	Inaba	0.88	U-3796	Ogawa	0.37
T-19765	Ogawa	0.81	T-19479	Inaba	0.36
395	Ogawa	0.70	U-11972	Ogawa	0.34
H-23448	Inaba	0.54	U-10570	Inaba	0.34
KB 356	Ogawa	0.50	P-20695	Inaba	0.32
48	Inaba	0.24	U-10600	Inaba	0.21
27447	Inaba	0.21	U-10875	Inaba	0.12
			U-11200	Inaba	0.09

[a] Strains KB 5, KB 356, KB 357, and KB 358 were obtained from G. Schmidt of the Max-Planck-Institut für Immunbiologie, Freiburg, Federal Republic of Germany. Strain 395 was isolated by and obtained from R. B. Sack of The Johns Hopkins University, Baltimore, Md. Strain 569B was isolated by the late N. K. Dutta of India. The remaining strains were obtained from the collection of the International Centre for Diarrhoeal Disease Research. *V. cholerae* strains were cultured in 3% peptone-water (pH 7.4).

[b] Enzyme activity is expressed as micromoles of *N*-acetylneuraminic acid (NANA) released from bovine submaxillary mucin per milliliter of the culture filtrate per unit of cell turbidity measured at 600 nm.

activity, which was found to be absent. Besides, the culture supernatant was neuraminidase negative.

To determine whether neuraminidase produced by various *V. cholerae* O1 and non-O1 strains had similar sugar specificity, *N*-acetylneuraminosyl (α-2→3) lactose was incubated with the dialyzed culture supernatant of *V. cholerae* 569B (classical, Inaba), *V. cholerae* 395 (classical, Ogawa), *V. cholerae* 18600 (El Tor, Ogawa), *V. cholerae* 10570 (El Tor, Inaba), non-O1 *V. cholerae* 6892, and non-O1 *V. cholerae* 3032. All these culture supernatants cleaved the trisaccharide, and the released product (lactose) was detected by paper chromatography with isopropanol-acetic acid-water (3:1:1) as the running solvent (17).

To determine the kinetics of toxin production, the culture filtrates obtained at various hours of growth of the bacteria were inoculated into ligated ileal loops of rabbits. No fluid accumulation was observed before 6 h (Fig. 1). There was a sharp increase in fluid accumulation by 7 h, followed by a gradual increase. Neuraminidase became detectable in the culture filtrate as early as 4 h, and it was produced in significant amounts at 5 h. It increased further until 6 h and then reached a plateau.

The ability to produce neuraminidase was examined in several strains of enteric bacteria, such as *V. cholerae* O1 (23 strains), *V. cholerae* non-O1 (21 strains), *V. parahaemolyticus* (16 strains), *A. hydrophila* (8 strains), enterotoxigenic *E. coli* (7 strains), and *Shigella* spp. (6 strains). *V. cholerae* O1 strains consistently produced neuraminidase. About one-third of all *V. cholerae* non-O1 strains tested secreted neuraminidase. Only two of eight strains of *A. hydrophila* were neuraminidase positive. None of the isolates of *V. parahaemolyticus*, enterotoxigenic *E. coli*, and *Shigella* spp. produced any detectable neuraminidase in the culture supernatant.

It has been reported that *V. cholerae* non-O1 strains produce neuraminidase (12), but such a study was based upon the examination of only one strain. When the study was extended to include several strains, we found that not all non-O1 strains were neuraminidase positive. Only one-third

TABLE 2. Specific activity of neuraminidase produced by *V. cholerae* strains

Strain	*V. cholerae* type	Sp act[a]
569B	O1, classical, Inaba	6.20
395	O1, classical, Ogawa	4.88
H-23448	O1, classical, Inaba	3.72
18600	O1, El Tor, Ogawa	1.54
T-19479	O1, El Tor, Inaba	1.07
U-11972	O1, El Tor, Ogawa	1.05
7193	Non-O1	1.04
8602	Non-O1	0.83
3032	Non-O1	0.68
6892	Non-O1	0.48

[a] Enzyme activity is expressed as micromoles of *N*-acetylneuraminic acid released from bovine submaxillary mucin per minute per milligram of protein upon incubation of the enzyme sample for a period of 10 min at 37°C.

TABLE 3. Influence of growth medium on the production of neuraminidase by *V. cholerae* O1 strains

Strain	*V. cholerae* type	NANA released (μmol)[a]	
		Peptone-water	Synthetic[b]
T-19767	Classical, Ogawa	0.91	0.26
569B	Classical, Inaba	0.88	0.54
395	Classical, Ogawa	0.70	0.42
18600	El Tor, Ogawa	0.46	0.31
T-19479	El Tor, Inaba	0.36	0.32
U-10570	El Tor, Inaba	0.34	0.09

[a] Enzyme activity is expressed as micromoles of *N*-acetylneuraminic acid (NANA) released from bovine submaxillary mucin per milliliter of the culture filtrate per unit of cell turbidity measured at 600 nm.

[b] Synthetic medium was prepared by a procedure described previously (10).

TABLE 4. Neuraminidase activity (secreted and cell bound) in *V. cholerae* and *E. coli*[a]

Test organism	Characteristics	NANA (μg)[b]		
		Culture filtrate	Polymyxin B released	Sonication released
V. cholerae O1				
395	Classical, Ogawa	142.80	0.80	0.10
W-13093	El Tor, Ogawa	95.20	0.80	0.00
48	Classical, Inaba	59.00	0.29	0.12
U-11200	El Tor, Inaba	23.00	0.12	0.07
E. coli				
339	LT^{+}[c]	0.00	0.00	0.00
403	LT^{+}	0.00	0.00	0.00

[a] Enzyme activity is expressed as micrograms of *N*-acetylneuraminic acid (NANA) released from bovine submaxillary mucin per milliliter of the test material per unit of cell turbidity measured at 600 nm.

[b] No neuraminidase activity was detected when cells were treated only with PBS.

[c] LT, Heat-labile toxin.

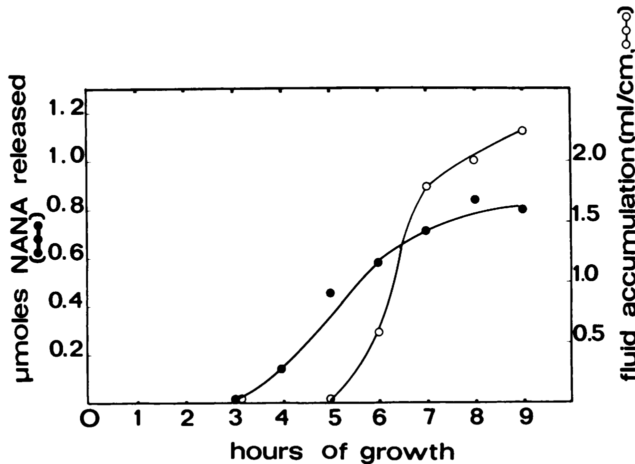

FIG. 1. Neuraminidase and toxin production during the growth of *V. cholerae* 569B (classical, Inaba). Culture filtrates were assayed for neuraminidase by the thiobarbituric acid test (2) and for biologically active toxin by the rabbit intestinal loop method as described by De and Chatterje (6). The fluid accumulation in the intestinal loops is expressed by calculating the ratio of the volume of the accumulated fluid (in milliliters) to the length of the intestinal piece (in centimeters). NANA, *N*-acetylneuraminic acid.

of them produced the enzyme. However, there is a similarity between the neuraminidase produced by *V. cholerae* O1 (classical and El Tor) and non-O1 strains, as the neuraminidase secreted by these strains cleaved (α-2→3) linkage of neuraminosyllactose. Earlier, Ohashi et al. (14) and Zinnaka and Carpenter (19) reported that the culture filtrates of some non-O1 strains secreted a permeability factor which was immunologically related to chlorea enterotoxins produced by *V. cholerae* O1 strains. These results suggest that *V. cholerae* O1 and non-O1 strains release some biological products which may be similar in nature.

The precise role of neuraminidase in the pathogenicity of *V. cholerae* has not been established. Diarrhea is caused by the secreted cholera toxin, and neuraminidase possibly plays a role in the induction of the disease. There is a close relationship between neuraminidase production and the induction of diarrhea. Clinically, classical strains induce the disease in a larger percentage of infected persons than do El Tor strains. We found that classical strains, in general, produced neuraminidase in larger quantities than did El Tor strains. In addition, the specific activity of the enzyme secreted by classical strains was higher than that produced by El Tor strains.

V. cholerae neuraminidase splits off terminal neuraminic or sialic acids of glycoproteins and glycolipids (8). When acting on gangliosides, it removes sialyl residues from major oligosialogangliosides, giving rise to monosialoganglioside GM1, which was proved to be resistant to the enzyme action in all experimental conditions hitherto tested. GM1 is known to be the receptor for cholera toxin (5, 18). Earlier, Staerk et al. (16) observed that when canine intestinal loops were pretreated with neuraminidase, there was an increase in the output of fluid after an incubation with cholera toxin. It is most likely that neuraminidase, after being released by the bacteria, attacks intestinal glycoproteins and gangliosides, thus unmasking receptor sites for cholera toxin.

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