

BIOLOGICAL SIMILARITY OF ENTEROTOXINS OF *VIBRIO CHOLERAE* SEROTYPES OTHER THAN TYPE 1 TO CHOLERA TOXIN AND *ESCHERICHIA COLI* HEAT-LABILE ENTEROTOXIN

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SUMMARY. *Vibrio cholerae* serotypes other than type 1, the so-called NAG vibrios, have been recognised as an important cause of diarrhoea. A few of them have been shown to produce an enterotoxin similar, immunologically and physiologically, to cholera toxin; and cholera toxin has been shown to be structurally, functionally and immunologically identical with *Escherichia coli* heat-labile toxin (LT). The present investigation has demonstrated biological similarities among cholera toxin, *E. coli* LT and enterotoxins produced by strains of *V. cholerae* of 59 serotypes other than 1, in the biological models, rabbit ileal loops and rabbit skin. Culture filtrates of almost all the strains were neutralised completely and all filtrates showed some neutralisation, in enterotoxic action and increase of permeability, by cholera antitoxin and *E. coli* LT antiserum. The partial neutralisation observed in a few strains was probably due to high concentrations of identical toxin rather than the presence of other toxic substances.

INTRODUCTION

The noncholera vibrios (McIntyre *et al.*, 1965) or the nonagglutinable vibrios (Felsenfeld, 1967), recently designated *Vibrio cholerae* serotypes other than 1 (Hugh and Feeley, 1972) have been isolated from various sources in nature (Barua, 1974; Sanyal *et al.*, 1974; Müller, 1977) as well as from healthy man (Marwah *et al.*, 1975). At first these bacteria were not considered very important in the causation of diarrhoea in man, but during the past two decades they were frequently being isolated from individual cases and during outbreaks of choleraic diarrhoea throughout the world (Yajnik and Prasad, 1954; Gaines *et al.*, 1964; McIntyre *et al.*, 1965; Smith and Goodner, 1966; Aldová *et al.*, 1968; Chatterjee, Gorbach and Neogy, 1970; Ko, Lutticken and Pulverer, 1973; Bäck, Ljunggren and Smith, 1974; Singh *et al.*, 1975; Tiwari *et al.*, 1975; Blake *et al.*, 1977; Dráskovičová, Karolček and Winkler, 1977; Hughes *et al.*, 1978; Nănescu and Ciufecu, 1978). A few strains of NAG vibrios were shown to produce an enterotoxin biologically and immunologically similar to cholera

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toxin (Zinnaka, Fukuyoshi and Okamura, 1972). Recently it has been shown that the cholera toxin and *Escherichia coli* heat-labile enterotoxin (LT) are structurally, functionally and immunologically identical (Clements, Yancey and Finkelstein, 1980). Because systematic study had not yet been made of the interrelationship among the enterotoxins elaborated by the different serotype strains of NAG vibrios, cholera toxin and *E. coli* LT, this investigation was carried out in the biological models, rabbit-gut loop and rabbit skin.

MATERIALS AND METHODS

Bacteria. Strains of the so-called NAG vibrios (*V. cholerae*, serotypes 2-60) were provided by Dr R. Sakazaki of the National Institute of Health, Japan. They were maintained in peptone-agar stab cultures at room temperature.

Antisera. Purified lyophilised cholera toxin, produced by *V. cholerae* strain 569B, was obtained from Dr S. Varallyay of the Swiss Serum and Vaccine Institute, Berne. For the preparation of antiserum to cholera toxin, albino rabbits (Belgian strain) weighing 3.0 kg were immunised with 50 µg of cholera toxin in 0.5 ml of phosphate-buffered saline (PBS, pH 7.2, 0.04M), mixed with 0.5 ml of Freund's incomplete adjuvant, intracutaneously in three doses at 14-day intervals, followed by two intravenous injections of 50 µg each, without adjuvant, at 7-day intervals. The rabbits were bled 7-10 days after the last injection. The crude serum was used as antitoxin. Normal serum collected before immunisation was the control.

Antiserum to *E. coli* LT, prepared with *E. coli* strain P263, was supplied by Dr F. Dorner of Sandoz Forschungsinstitut, Vienna.

Preparation of culture filtrates. Culture filtrates of the different serotype strains of the NAG vibrios were prepared by the method of Dubey and Sanyal (1978). The bacteria were plated on nutrient agar from stock cultures. After overnight incubation, five or six smooth colonies from each strain were picked and inoculated into 50 ml of sterile Brain Heart Infusion Broth (BHIB, Difco) in 250-ml conical flasks. The cultures were incubated at 37°C in a shaking water bath (120 oscillations/min) for 18 h and were then centrifuged at 4°C for 30 min at 22 000 *g*; the supernates were decanted, passed through Millipore membrane filters of 0.22 µm average pore diameter and preserved at -20°C until use. Some of the strains gave a very poor yield of toxin initially and so were given one or two serial passages in rabbit ileal loops as in the method of Singh and Sanyal (1978).

Ileal-loop tests. Ligated adult rabbit ileal-loop tests were performed by the method of De and Chatterje (1953) as modified by Annapurna and Sanyal (1977) and in albino rabbits (Belgian strain) weighing 1.5-2.0 kg. In all the experiments, 1 ml of sterile BHIB served as negative control and 0.5 ml of culture filtrate of a particular strain mixed with 0.5 ml of normal rabbit serum served as positive control. All the experiments were done in duplicate and the mean values were adopted.

Neutralisation of enterotoxic activity of culture filtrates of NAG vibrios by cholera antitoxin or by *E. coli* LT antitoxin was investigated by the method of Kasai and Burrows (1966) and Dubey and Sanyal (1979). The cholera antitoxin, or the *E. coli* LT antitoxin, and the normal rabbit serum were inactivated at 56°C for 30 min. Serial twofold dilutions of the antiserum with sterile PBS were mixed in 1.0-ml volumes with 1.0 ml of culture filtrate of a particular serotype strain and 1.0 ml of the same culture filtrate was also mixed with 1.0 ml of undiluted normal rabbit serum. The mixtures were then incubated at 37°C for 1 h and injected immediately, one in each loop in 1-ml volume. The animals were killed after 6 h and the volume of fluid and the length of ileal loops were measured to calculate the accumulation of fluid/unit length.

Tests in rabbit skin. Neutralisation of permeability factor (PF), induration and dermonecrotic activity of the culture filtrates of NAG vibrios by cholera and *E. coli* LT antitoxins were investigated by the methods of Craig (1971), Evans, Evans and Gorbach (1973) and Dubey and Sanyal (1978). The hair from the body of the adult rabbits was removed as far as possible. Areas of approximately 30 mm diameter were marked and each area was given a number. Normal rabbit serum and the antitoxins were inactivated and the toxin-antitoxin mixtures were prepared as described for the neutralisation tests in ileal loops. The materials were then injected

intracutaneously in 0.1 ml volume at the centre of each marked site. After 18 h the diameters of the areas of induration were measured and areas showing necrosis were noted. Evans blue (2% in 0.15M sodium chloride) was then injected intravenously in a dose of 60 mg/kg body weight. The diameters of the blue zones were measured 1 h after injection of the dye. All experiments were done in duplicate and the mean values were adopted.

RESULTS

Rabbit ileal-loop assays

The dilution of cholera antiserum that completely neutralised the activity of a culture filtrate of *V. cholerae* strain 569B in ligated ileal loops was found to be 1 in 64. Therefore antitoxin dilutions as far as 1 in 64 were used in the neutralisation tests of the culture filtrates of the NAG vibrios.

The neutralisation of enterotoxic activity of culture filtrates of the different serotype strains of NAG vibrios with cholera antitoxin is shown in table 1 and in figs. 1 and 2. Of the 59 culture filtrates of the different serotypes, 35 were completely neutralised and 24 were only partially (76–99%) neutralised when tested with undiluted antitoxin. Complete neutralisation of 32, 25 and 14 culture filtrates was noted at antitoxin dilutions 1 in 2, 1 in 4 and 1 in 8, respectively. There was proportionately less neutralisation of enterotoxic activity with serial twofold dilutions of the antitoxin. None of the culture filtrates, however, was completely neutralised by the antitoxin diluted to 1 in 16 or more. Accumulation of fluid ranged between 0.1 and 1.0 ml/cm of gut after neutralisation tests of culture filtrates, depending on the antitoxin dilution used. In none of the experiments was the culture filtrate neutralised by normal rabbit serum. The range of fluid accumulated in the positive control loops was 0.5–1.25 ml/cm with a mean value of 0.9 ± 0.25 ml/cm. Haemorrhagic fluid was noted in some of the control loops.

Neutralisation of enterotoxic activity of culture filtrates of different serotype strains of NAG vibrios with *E. coli* LT antitoxin is shown in table II and fig. 3. Of the culture filtrates of 18 randomly selected serotypes that were tested, 12 were completely neutralised, three were neutralised by more than 75% and the remaining three were neutralised by more than 50% of their enterotoxic activity by undiluted antitoxin.

TABLE I

Neutralisation of enterotoxicity of culture filtrates of NAG vibrios by different dilutions of cholera antitoxic serum

Percentage neutralisation	Number of culture filtrates neutralised by antiserum at a dilution of 1 in						
	1	2	4	8	16	32	64
100	35	32	25	14	0	0	0
76–99	24	27	34	25	15	4	0
51–75	0	0	0	20	34	11	0
26–50	0	0	0	0	10	32	0
< 25	0	0	0	0	0	12	59

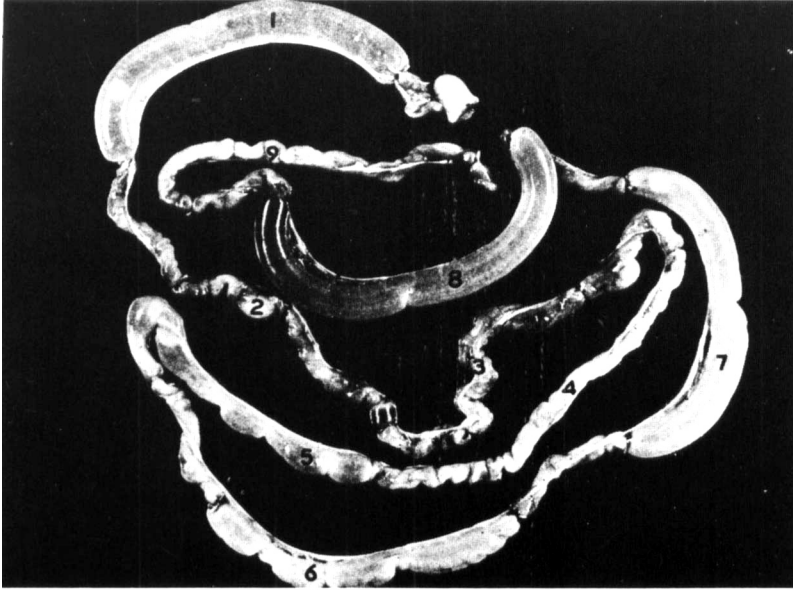


FIG. 1.—Neutralisation of culture filtrate of NAG vibrio by cholera antitoxin in ileal loops. Loop 1: positive control—0.5 ml of culture filtrate mixed with 0.5 ml of normal rabbit serum. Loops 2–8: 0.5 ml of culture filtrate mixed with 0.5 ml of undiluted, 1 in 2, 1 in 4, 1 in 8, 1 in 16, 1 in 32 and 1 in 64 dilutions of cholera antiserum. Loop 9: negative control—1 ml of sterile broth.

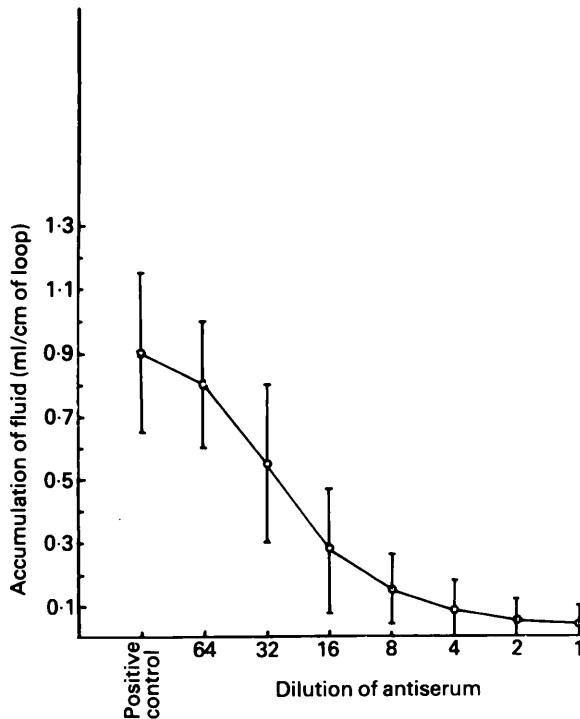


FIG. 2.—Accumulation of fluid/unit length of loop (mean \pm SD) after neutralisation of culture filtrates of NAG vibrios by cholera antitoxin.

TABLE II

Neutralisation of enterotoxicity of culture filtrates of NAG vibrios by different dilutions of *E. coli* LT antitoxic serum

Percentage neutralisation	Number of culture filtrates neutralised by antiserum at a dilution of 1 in						
	1	2	4	8	16	32	64
100	12	11	4	0	0	0	0
76-99	3	4	9	9	0	0	0
51-75	3	0	2	5	10	0	0
26-50	0	3	3	4	6	9	0
< 25	0	0	0	0	2	9	18

There was a proportional decrease in neutralisation of enterotoxic activity of the culture filtrates with serial twofold dilution of the antitoxin. None of the culture filtrates tested was completely neutralised by the antitoxin diluted 1 in 8 or more. After neutralisation tests, accumulation of fluid/cm of gut ranged from less than 0.1 ml/cm to 1.2 ml/cm, depending on the antitoxin dilutions used. On average the accumulation of fluid in the positive control loops was 0.9 ± 0.25 ml/cm with a range of 0.5-1.25 ml/cm of gut.

Rabbit-skin assays

Three factors were taken into account in measuring the reactivity in rabbit skin of the culture filtrates before and after neutralisation: skin blueing factor or vascular permeability factor, induration factor and necrosis.

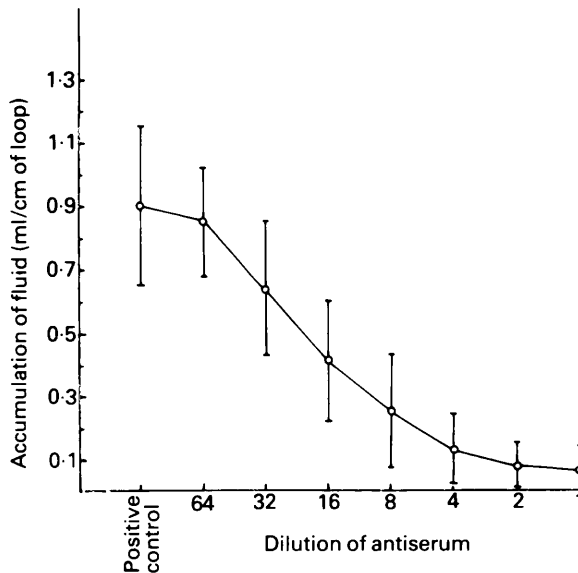


FIG. 3.—Accumulation of fluid/unit length of loop (mean \pm SD) after neutralisation of culture filtrates of NAG vibrios by *E. coli* LT antitoxin.

TABLE III

Neutralisation of permeability-factor activity of culture filtrates of NAG vibrios by different dilutions of cholera antitoxic serum

Percentage neutralisation	Number of culture filtrates neutralised by antiserum at a dilution of 1 in				
	1	2	4	8	16
100	17	11	7	4	0
76-99	1	1	1	0	0
51-75	6	8	8	5	0
26-50	1	4	5	12	4
<25	0	1	4	4	21

Neutralisation of the activity in rabbit skin of culture filtrates of different serotypes of NAG vibrios by cholera antitoxin is shown in table III and fig. 4. When undiluted antitoxin was used, complete, 87%, 51-75% and 50% neutralisation of PF activity was seen in 17, 1, 6 and 1 culture filtrates respectively of 25 randomly selected serotypes that were tested (table III). Proportionately less neutralisation of PF activity was observed after serial dilution of antitoxin (fig. 4). None of the filtrates showed full neutralisation of PF activity when an antitoxin dilution of 1 in 16 was used.

Four of the filtrates did not produce any induration in the control itself. Of the remaining 21 filtrates, 11 were completely neutralised with undiluted antitoxin,

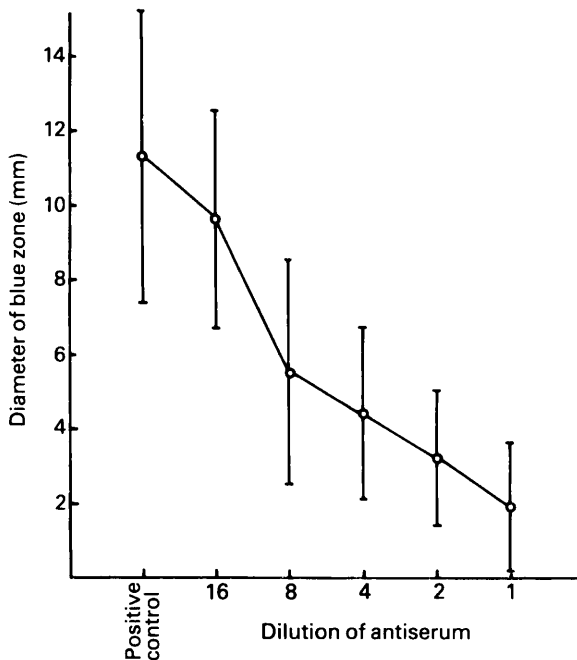


FIG. 4.—Neutralisation of vascular-permeability factor of culture filtrates of NAG vibrios by cholera antitoxin (mean \pm SD).

TABLE IV

Neutralisation of induration-factor activity of culture filtrates of NAG vibrios by different dilutions of cholera antitoxin

Percentage neutralisation	Number of culture filtrates neutralised by antiserum at a dilution of 1 in				
	1	2	4	8	16
100	11	8	8	5	0
76-99	0	0	0	1	0
51-75	8	6	1	1	0
26-50	2	6	11	13	5
<25	0	1	1	1	16

whereas 10 were only partially neutralised (table IV). With increasing dilutions of antitoxin there was a proportional decrease in neutralisation of the induration effect (fig. 5).

Necrosis was not noted in nine control reactions among 25 filtrates. In the case of the remaining 16 filtrates the necrosis factor was completely neutralised by undiluted antitoxin, and in four even at a dilution of 1 in 16.

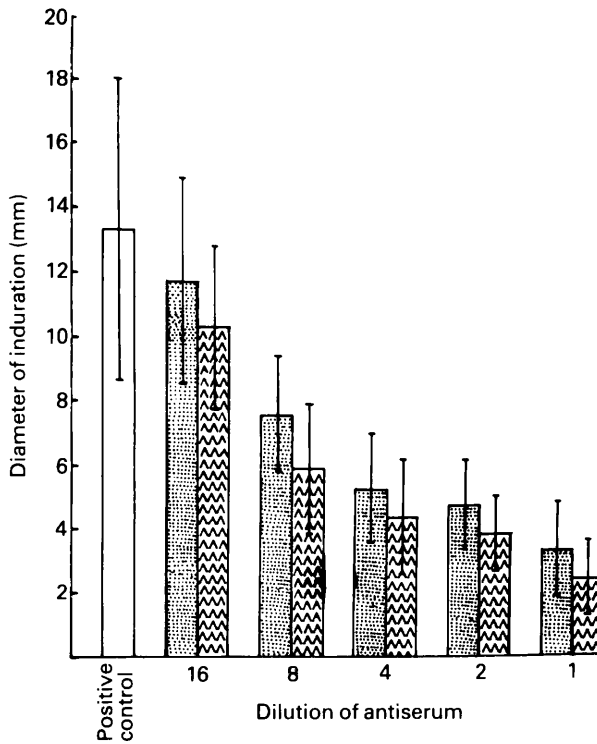


FIG. 5.—Neutralisation of induration factor of culture filtrates of NAG vibrios by cholera and *E. coli* LT antitoxins (mean \pm SD). \square = Filtrate with normal rabbit serum; \blacksquare = filtrate with *V. cholerae* antiserum; \boxtimes = filtrate with *E. coli* antiserum.

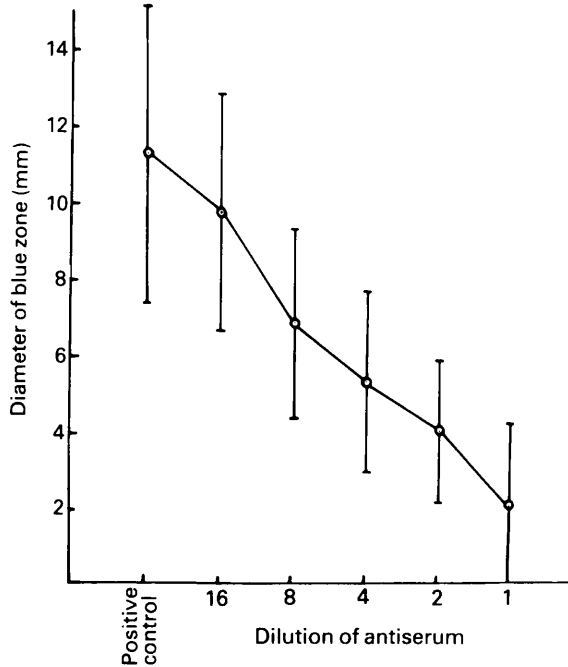


FIG. 6.—Neutralisation of vascular-permeability factor of culture filtrates of NAG vibrios by *E. coli* LT antitoxin (mean \pm SD).

Neutralisation of activity in rabbit skin of culture filtrates by *E. coli* LT antitoxin is shown in table V and fig. 6. Of the filtrates from 22 randomly selected serotypes that were tested, 13 were completely neutralized in PF activity and 9 were partially neutralised by undiluted antitoxin (table V). There was proportional decrease in neutralisation of skin PF with serial dilutions of antiserum (fig. 6).

Of the 22 filtrates, four did not cause skin induration. Of the remaining 18, complete neutralisation was seen in 8, and the rest were partially neutralised by undiluted antitoxin (table VI). A proportional decrease in neutralisation of the

TABLE V

Neutralisation of PF activity of culture filtrates of NAG vibrios by different dilutions of *E. coli* LT antitoxin

Percentage neutralisation	Number of culture filtrates neutralised by antiserum at a dilution of 1 in				
	1	2	4	8	16
100	13	6	3	2	0
76-99	1	1	0	0	0
51-75	6	8	7	3	0
26-50	2	6	8	7	3
< 25	0	1	4	10	19

TABLE VI

Neutralisation of induration-factor activity of culture filtrates of NAG vibrios by different dilutions of *E. coli* LT antitoxin

Percentage neutralisation	Number of culture filtrates neutralised by antiserum at a dilution of 1 in				
	1	2	4	8	16
100	8	6	6	0	0
76-99	2	1	0	1	0
51-75	3	4	5	5	0
26-50	5	6	3	5	0
<25	0	1	4	6	18

induration factor was observed on serial dilution of the antitoxin (fig. 5). Necrosis was noted with 14 of the 18 culture filtrates, and was neutralised by dilutions of antiserum to 1 in 16.

DISCUSSION

The majority of the culture filtrates of the so-called NAG vibrios tested in ileal-loop assay were completely neutralised by antitoxin to *V. cholerae* strain 569B. Only 19 of 59 filtrates were not completely neutralised; in these cases the filtrates might contain more toxin molecules than could be completely neutralised by the available antitoxin. Alternatively, it has been shown that NAG vibrios elaborate a large amount of lipopolysaccharide in culture medium (Holmgren, Lönnroth and Ouchterlony, 1971; Pike and Chandler, 1969) and the LPS might interfere with neutralisation of the enterotoxin. *V. cholerae* serotype 1 has also been shown to produce different enzymes along with the enterotoxin; some of these factors, produced by NAG vibrios, might have an additive effect in the enterotoxic activity and, of course, an antitoxin to pure cholera toxin would be unable to neutralise such a factor. All the filtrates tested were very poorly neutralised by the antitoxin diluted 1 in 64, while the neutralisation titre with the homologous toxin was 64. Spira and Daniel (1980) while working with several strains of non-O group-1 *V. cholerae* also noticed a reaction of partial to complete identity with cholera toxin in neutralisation tests with homologous and heterologous antisera. The present study indicates that the enterotoxins produced by different serotypes of NAG vibrio are biologically similar to cholera toxin.

Structural, functional and immunological identities between cholera toxin and *E. coli* LT have been established (Gyles, 1974; Clements and Finkelstein, 1978; Clements *et al.*, 1980). Klipstein and Engert (1977) also demonstrated inhibition of action of cholera toxin on rat jejunum by antisera raised against the LT preparations of *E. coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae*, suggesting antigenic relatedness among these enterotoxins. The biological relationship between *E. coli* LT and enterotoxins elaborated by NAG vibrios has not previously been investigated. In this study, complete neutralisation of enterotoxic activities of the majority of the culture filtrates with antitoxin to *E. coli* LT indicated that the toxins are identical in biological activity. Partial neutralisation of some of the culture filtrates might be due either to the

presence of more toxin molecules in them than could be neutralised by the available titre of the antitoxin or to the presence of other biologically active factors, immunologically unrelated to *E. coli* LT, such as enzymes.

In the neutralisation tests of PF activities with cholera antitoxin, most of the filtrates tested were completely neutralised, indicating the antigenic similarity between these toxins. Partial neutralisation was observed in some which might have been due to elaboration of more toxin by a particular strain, as explained earlier. Oashi *et al.* (1972) and Zinnaka and Carpenter (1972) also observed similar results while working with only a few strains of NAG vibrios. However, Zinnaka, Fukuyoshi and Okamura (1972) demonstrated complete neutralisation of the PF activity of four NAG vibrio toxins by cholera antitoxin, after purification in aluminium hydroxide gel. This observation ruled out the possibility of a second toxin factor being present, as had been suggested by Oashi *et al.* (1972). The PF activities of most of the culture filtrates were also neutralised by antitoxin to *E. coli* LT, showing a relationship amongst them in regard to their activity in increasing permeability.

The induration factor was not constantly associated with PF activity, indicating that it may not be related to enterotoxin. Cholera antitoxin could neutralise the induration factor of the NAG culture filtrates, though less efficiently than it neutralised PF. After neutralisation tests with cholera antitoxin, residual induration in the absence of blueing indicated the possible presence of other substances released extracellularly by the NAG vibrios. Dubey and Sanyal (1978) also reported less efficient neutralisation of the induration than the PF produced by enterotoxin of *Aeromonas hydrophila*, by homologous antiserum. Finkelstein *et al.* (1976) identified a blanching factor in *E. coli* LT which could produce dermal thickening and necrosis. Presence of such a factor in enterotoxins elaborated by NAG vibrios could not be excluded. The induration was not completely neutralised by antitoxin to *E. coli* LT, and therefore the blanching factor, if present in NAG culture filtrates, is only partially related to that of *E. coli* LT.

The factors producing necrosis do not seem to be related to enterotoxin, because necrosis was often absent even when there was a large blueing zone. Dermonecrosis was also seen with cholera toxin (Craig, 1965) and the enterotoxin of *A. hydrophila* (Ljungh, Popoff and Wadström, 1977). Although the dermonecrotic factor was almost always neutralised by cholera antitoxin and by antitoxin to *E. coli* LT, the exact relationship between this factor and enterotoxin is not known.

All these data indicate that the observed differences in toxigenicity of NAG vibrios are quantitative rather than qualitative. Enterotoxins of *V. cholerae* serotypes other than 1 are identical with *V. cholerae* type-1 enterotoxin and similar to *E. coli* LT in their biological activity.

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