Immunobiological relationships of the enterotoxins produced by cholera toxin gene-positive (CT+) and -negative (CT−) strains of Vibrio cholerae O1

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Summary. The optimum rabbit ileal loop (RIL) reacting doses of the new cholera toxin (NCT) produced by cholera toxin gene-negative (CT−) strain X-392 and of the enterotoxin produced by cholera toxin gene-positive (CT+) strain 569B of Vibrio cholerae O1 were found to be 32 μg and 22 μg respectively. Production of NCT by the CT+ strain, in addition to CT, was confirmed by in-vivo neutralisation tests. Anti-569B-enterotoxin neutralised the optimum RIL reacting activity of NCT completely at 1 in 16 dilution, whereas the activity of 569B enterotoxin was only partially neutralised (44%) by anti-NCT. Similarly, partial neutralisation (66%) was observed when purified anti-CT was mixed with 569B enterotoxin. Therefore, the fluid accumulation produced in the RIL by 569B enterotoxin was the combined effect of both CT and NCT. No antigenic relationship between NCT and CT could be demonstrated in gel-diffusion tests.

Introduction

Cholera toxin (CT) has long been recognised as the most important of all the extracellular products of Vibrio cholerae O1 in the pathogenesis of the disease caused by this organism. The enterotoxins (i.e. CTs) produced by the two main serogroups, Inaba and Ogawa, have been shown to be immunologically identical.1 In 1983, Sanyal et al.2 demonstrated that live oral vaccine strains of CT gene-negative (CT−) V. cholerae O1 produce a new cholera toxin (NCT) that causes a secretory response in various animal models2,3 and is probably the cause of diarrhoea in human volunteers4 who ingest the vaccine strains, such as A−B+ or A+B− mutants.5

The rabbit ileal loop (RIL) reacting activity of this NCT could not be neutralised by antiserum against purified CT. Furthermore, monoclonal antibodies against CT and its A and B subunits were unable to neutralise the skin-permeability-factor activity of this toxin.3 Analysis with Ouchterlony's gel-diffusion test demonstrated that diarrhoeal isolates of CT gene-positive (CT+) V. cholerae O1 Ogawa, and the highly toxigenic Inaba strain 569B, also produce NCT.6 The NCTs produced by CT− V. cholerae O1 strains of diverse origin have recently been shown to be immunobiologically identical.7 The present study was undertaken to measure the enterotoxic activity of 569B toxin in terms of optimum RIL reacting dose, to examine whether anti-569B-enterotoxin can neutralise in vivo the secretogenic activity of NCT preparations from CT− strains, to determine the extent of neutralisation of 569B enterotoxin in vivo by anti-NCT and by purified anti-CT, and to examine the antigenic relationship of NCT to purified CT in vitro.

Materials and methods

Strains of V. cholerae O1

CT− strains X-392 and 1074-78 (environmental isolates) and 2740-80 (diarrhoeal isolate) were provided by J.B. Kaper, Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, USA. The highly toxigenic Inaba strain 569B was from our laboratory stock.

Preparation of enterotoxins and antitoxins

Enterotoxin of CT+ strain 569B was prepared in syncape medium8 by the method of Saha and Sanyal.6,7 In brief, 1 L of culture was incubated overnight at 37°C, with constant shaking (120 oscillations/min), and centrifuged at 4°C; the supernate was filtered through a membrane of 0.22 μm average pore diameter. The filtrate was
saturated gradually with recrystallised ammonium sulphate to 80% at 4°C, and kept overnight at 4°C; the precipitate was then collected by centrifugation (20 000 g for 30 min) at 4°C, dissolved in 10 ml of 0.02 M phosphate buffered saline (PBS), pH 7.2, and dialysed against the same buffer with 6-8 changes to remove the ammonium sulphate completely. The dialysate was then passed through a 0.22-μm membrane filter and divided into small amounts for storage at -10°C. This filtrate was then centrifuged (20 000 g), and kept overnight at 4°C; the precipitate was then collected by centrifugation (20 000 g)

The protein content of which was estimated by the method of Lowry.

preparations, each of which had been adjusted to a protein concentration of 64 μg/ml of PBS.

The optimum RIL reacting dose of 569B enterotoxin was found to be 22 μg of protein; this was mixed, as above, with dilutions of anti-569B-enterotoxin, anti-NCT or anti-CT, to determine their neutralising activity.

In all these tests, a mixture of enterotoxin and normal rabbit serum was used as a positive control, and PBS as a negative control. Three RILs were used for the determination of each volume/length ratio.

**Immunodiffusion test**

To confirm the antigenic relationship between CT and NCT, Ouchterlony's immunodiffusion test was performed with the purified anti-CT against NCT, against 569B enterotoxin, and against purified CT.

**Results**

**Optimum RIL reacting dose of 569B enterotoxin**

Assay in RILs showed that 22 μg of 569B enterotoxin caused maximum accumulation of fluid, 1.25 ml/cm; and no further increase was observed with higher doses.

**Neutralisation of 569B enterotoxin by homologous antitoxin**

The highest dilution of anti-569B-enterotoxin that completely neutralised the RIL reacting activity of homologous enterotoxin was 1 in 64 (fig. 1), and no neutralisation was observed when the enterotoxin was mixed with pre-immunisation serum. There was proportionately less neutralisation of the enterotoxic activity with higher dilutions of antitoxin.

**Neutralisation of enterotoxic activity of NCT by anti-569B-enterotoxin**

The optimum RIL reacting activity of the NCT from three CT strains was completely neutralised by anti-569B-enterotoxin at 1 in 16 dilution (fig. 1). As all three preparations were neutralised at the same dilution, the results of only one are shown. No neutralisation was observed in positive control loops.

**Neutralisation of 569B enterotoxin by anti-NCT and anti-CT**

The optimal RIL reacting activity of 569B enterotoxin was only partially neutralised by anti-
NCT. The neutralisation graph (fig. 2) showed maximum neutralisation up to 1 in 64 dilution, at which the accumulation of fluid was 0.70 ml/cm (44% neutralisation)—the same as when undiluted anti-NCT was used. Gradual increase in fluid accumulation was observed with higher dilutions of anti-NCT. Similarly (fig. 3) anti-CT also caused only partial neutralisation: accumulation of fluid was 0.43 ml/cm (66% neutralisation) at 1 in 128 dilution. There was proportionately less neutralisation with higher dilutions.

**Immunodiffusion test**

Both 569B enterotoxin and purified CT gave precipitin bands against anti-CT, showing a reaction of identity; no band was observed between the wells containing the NCT and anti-CT (fig. 4).
Discussion

The observation that less 569B enterotoxin (22 µg) was required to give a maximum reaction in the RIL than with the NCT preparation (32 µg) suggests that the presence of both CT and NCT in the former preparation exerted an additive effect in stimulation of similar fluid secretion with a smaller amount of toxin protein than that of NCT alone. The other possibility that CT is merely more active than NCT could, however, not yet be excluded.

The other possibility that CT is merely more active amount of toxin protein than that of NCT alone. The amount required to neutralise NCT may be greater because the gene responsible for NCT was not deleted. This hypothesis is substantiated by the occurrence of diarrhoea in the vaccinated volunteers.

Furthermore, in gel diffusion tests NCT did not give any precipitin band against purified anti-CT; and this substantiates the preliminary finding of Sanyal et al. that NCT is not related antigenically to CT. NCT was found to differ from CT also in receptor site, mode of action and genetic homology. Induction of antitoxic immunity against cholera may, therefore, be achieved only by the use of both CT and NCT.

Recent studies on immunity in cholera have shown long-lasting memory in the mucosal immune response. Oral B subunit and live cholera vaccines, therefore, may be potentially effective in immunisation; however, the present study suggests that the former may need to be combined with a non-toxic (toxoid) but protective form of NCT to give better protection. Oral or parenteral vaccine made with CT or its B subunit would not produce antibody against NCT; and this may explain the low efficacy of B subunit vaccine against cholera in the field study in Bangladesh. In a recent study strain 569B was used to prepare live oral candidate vaccine strains (CVD 103 and CVD 103 HgR; CT gene deleted) which did not produce shiga-like toxin or haemolysin. The present study indicates that strain 569B is also a NCT producer; and thus these candidate vaccine strains may have the potential to produce disease, because the gene responsible for NCT was not deleted. This hypothesis is substantiated by the occurrence of diarrhoea in the vaccinated volunteers.

Therefore, candidate vaccine strains should be devoid of the toxic form of both factors (CT and NCT); but for efficacy they should confer immunity against NCT as well as CT.

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REFERENCES


