

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 models^{2,3} 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 syncase 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

Received 14 Aug. 1989; accepted 26 Oct. 1989.
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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- μ m membrane filter and divided into small amounts for storage at -10°C. This filtrate constituted the enterotoxin preparation (569B enterotoxin), the protein content of which was estimated by the method of Lowry *et al.*⁹

NCT was prepared from the three CT⁻ strains by the same procedure.

Preparation of antitoxins. Albino rabbits (Belgium strain) weighing 2.5 kg were immunised with enterotoxin preparations from the CT⁻ strain X-392 and CT⁺ strain 569B by the method of Saha and Sanyal.^{6,7} The antisera were termed anti-NCT and anti-569B-enterotoxin respectively. Sera collected before immunisation served as negative controls.

Purified CT and anti-CT used in this study were kindly supplied by Ann-Marie Svennerholm and J. Holmgren, University of Göteborg, Sweden. The CT was prepared from the CT⁺ strain 569B. We have demonstrated the absence of NCT in this preparation earlier,⁶ and this was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis that showed only two bands corresponding to the A and B subunits, quite distinct from NCT.

Optimum RIL reacting dose of 569B enterotoxin

Graded doses of 569B enterotoxin, in terms of protein content, were inoculated into different loops of three rabbits. The animals were killed after 8 h and the accumulation of fluid (ml/cm of gut) was measured. The minimum amount of enterotoxin that caused the maximum amount of fluid accumulation was considered as the optimum RIL reacting dose.

Assay of NCT

The NCT preparations for the present study were assayed as above and had been used in our previous experiments.⁷

In-vivo neutralisation experiments

Attempts were made, by our previously described method in RILs,^{7,10,11} to neutralise the enterotoxic activity of the three NCT preparations from CT⁻ strains with anti-569B-enterotoxin, and to neutralise the activity of 569B enterotoxin with homologous antitoxin, with anti-NCT and with anti-CT.

NCT in 0.5-ml volumes containing 32 μ g of protein (i.e., the optimal dose as described earlier⁷) was added to each of a series of twofold dilutions of antitoxin in PBS. The mixtures were incubated in a water bath at 37°C for 30 min and thereafter tested in RILs to obtain the highest

dilution of anti-569B-enterotoxin that would cause complete neutralisation of enterotoxic activity of the NCT preparations, each of which had been adjusted to a protein concentration of 64 μ g/ml of PBS.

The optimum RIL reacting dose of 569B enterotoxin was found to be 22 μ g 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 μ g 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-

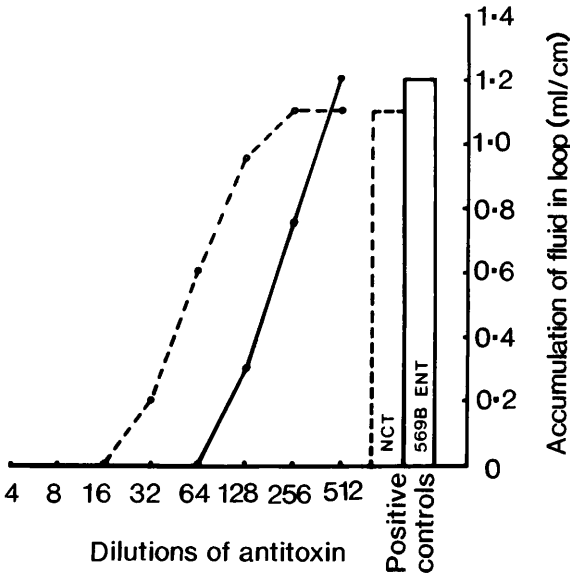


Fig. 1. Neutralisation of RIL reacting activity of NCT (----) (at 1 in 16 dilution), and 569B enterotoxin (—) (1 in 64 dilution), by anti-569B-enterotoxin.

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

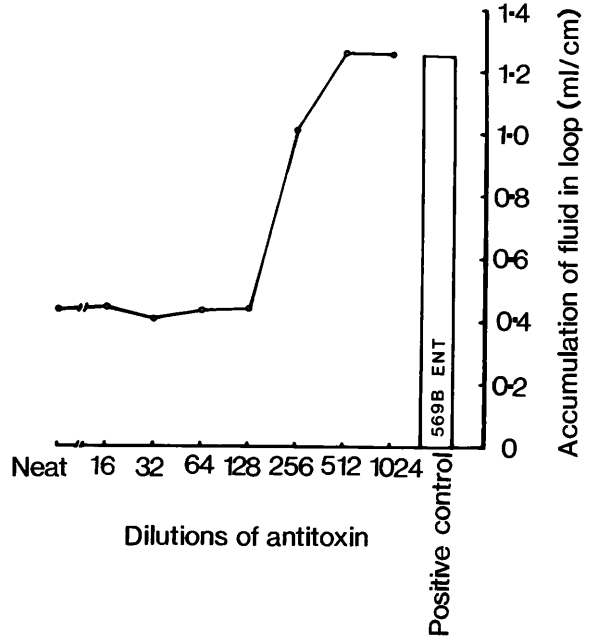


Fig. 3. Partial neutralisation of 569B enterotoxin by anti-CT.

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).

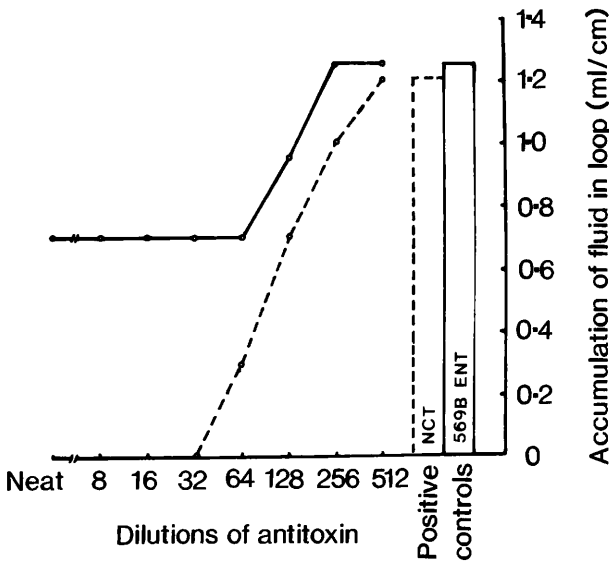


Fig. 2. Neutralisation of NCT (----) and 569B enterotoxin (—) by anti-NCT, showing complete neutralisation (at 1 in 32 dilution) and partial neutralisation respectively.

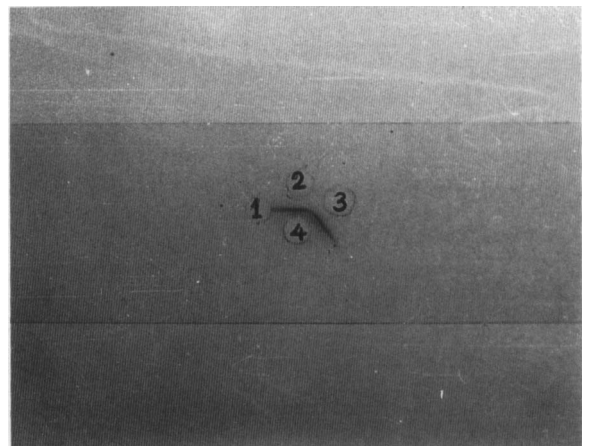


Fig. 4. Immunodiffusion of NCT (well 1), CT (well 2) and 569B enterotoxin (well 3) against anti-CT (well 4). CT and 569B enterotoxin give a precipitation line of identity, but there is no reaction between NCT and anti-CT.

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 data from in-vivo neutralisation experiments (fig. 1) showed that anti-569B-enterotoxin could neutralise the optimal RIL reacting activity (22 µg) of homologous enterotoxin completely at 1 in 64 dilution; and it also neutralised the enterotoxic activity of the NCT (32 µg), prepared from three CT⁻ strains of diverse origin, though only at a higher concentration (1 in 16). These data confirm the earlier observation¹¹ and indicate the presence of NCT neutralising antibody in anti-569B-enterotoxin. This suggests that antibody against crude 569B enterotoxin can protect against the secretory effect of both CT and NCT. The amount required to neutralise NCT may be greater because the optimal RIL reacting dose of 569B enterotoxin gave the combined effect of CT and NCT, and thus contained less of each toxin than in the optimal dose of CT or NCT alone.

Purified anti-CT could neutralise the 569B enterotoxin only partially (fig. 3). Similar partial neutralisation of the same enterotoxin was also observed with anti-NCT (fig. 2). These data indicate that the effect of 569B enterotoxin is due partly to CT and partly to NCT; and this reaffirms our previous finding that strain 569B produces NCT in addition to the classical CT.⁶ The data also suggest that diarrhoea may be caused by either CT or NCT or both, and they establish NCT as a potent enterotoxin.

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.*² 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.³ 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.^{12,13} 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.¹⁴

In a recent study¹⁵ 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 strains¹⁵ 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.

We are grateful to Mr S. K. Saha for his help during the experiments and Mr D. Venu Gopal for his secretarial assistance. The study was supported in part by the Council of Scientific and Industrial Research, New Delhi.

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