A non-haemagglutinating form of *Clostridium difficile* toxin A

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Summary. Analysis of crude culture filtrate of *Clostridium difficile* by Mono Q-anion exchange fast protein liquid chromatography (FPLC) demonstrated that toxin A had distinct peaks of activity for cytotoxicity and haemagglutination, as also did highly purified toxin A obtained by thyroglobulin affinity chromatography (TG) followed by two sequential anion-exchange chromatographic steps with Q-Sepharose FF and Mono Q. From TG unbound fractions a highly cytotoxic but weakly haemagglutinating variant (toxin A') of toxin A was obtained by Q-Sepharose FF and Mono Q chromatography. Analysis of toxins A and A' from cultures of *C. difficile* in a chemically defined medium, and of toxin A dialysed against brain heart infusion broth, indicated that A' was not merely toxin A coupled to a component of the growth medium. Polyacrylamide gel electrophoresis under non-denaturing conditions showed that toxins A and A' had the same M_r . Immunoblotting with mouse monospecific A antitoxin showed that five bands larger than the major 240-Kda band were more strongly developed in toxin A than in A' in denaturing but non-reducing conditions, and in reducing conditions eight bands (38–175 Kda) were seen in toxin A but not A'. Immunoblotting with a monoclonal antibody (PCG-4) showed that, in both reducing and non-reducing conditions, two bands of 160 and 155 Kda were more prominent in toxins A and A' respectively, and four bands (195, 180, 175 and 125 Kda) were detected only in toxin A'.

Introduction

Clostridium difficile, the causative agent of pseudo-membranous colitis (PMC) and many cases of antibiotic-associated diarrhoea,^{1–4} produces at least two toxins, designated A (enterotoxin) and B (cytotoxin). Toxin A probably causes most of the symptoms associated with PMC because (a) only toxin A induces extensive tissue damage and fluid response in experimental animals,^{5–7} and (b) the severity of the symptoms in antibiotic-associated ileo-caecitis in hamsters is more closely related to the in-vivo production of toxin A than of toxin B.⁸ However, toxins A and B act synergically.^{7,9}

Toxin A binds to brush border membranes of hamsters; it also haemagglutinates rabbit erythrocytes at 4°C but not at 37°C.¹⁰ The binding of toxin A to bovine thyroglobulin showed a similar dependence on temperature.¹⁰ The binding site was identified as a glycoprotein containing the non-reducing terminal sequence Gal α 1-3Gal β 1-4GlcNAc, but other Gal β 1-4GlcNAc-containing glycoproteins are thought to be the gastrointestinal mucus receptors for toxin A.¹¹ The temperature-dependent binding of toxin A to bovine thyroglobulin has recently been used to purify

toxin A,¹² though small amounts of toxin B and other proteins are present in the thermal eluent at 37°C.¹³ A modified method of purification consists of two sequential anion-exchange chromatographic steps following thyroglobulin affinity chromatography.¹⁴

Toxin A agglutinates rabbit erythrocytes; toxin B does not do so,^{10,13} although some authors disagree.¹⁵ In the present study, the relationship between cytotoxicity and the haemagglutinating activity of toxin A, and evidence for a non-haemagglutinating form of the toxin were examined.

Materials and methods

Bacterial strain and culture filtrate

C. difficile strain VPI 10463 (originally obtained from Dr T.D. Wilkins, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA) was kindly provided by Dr M. Giuliano (Laboratorio di Bacteriologia e Micologia Medica, Istituto Superiore di Sanita, Rome, Italy). Its growth in dialysis tubing and the preparation of culture filtrate were as described previously.¹³ After anaerobic incubation (18 h, 37°C) in 10 ml of Brain Heart Infusion Broth (BHI; Oxoid) containing yeast extract (Beta Lab, Surrey) 0.5% w/v, L-cysteine-HCl (BDH Chemicals) 0.05% and sodium

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formaldehyde sulphonylate 0.03%, the culture was centrifuged at 3670 *g* and 4°C and the pelleted bacterial cells were resuspended in 10 ml of phosphate-buffered saline (PBS), pH 7.2; 2 ml of this suspension were inoculated into dialysis tubing containing c. 100 ml of PBS which had been equilibrated overnight against BHI. After anaerobic incubation for 5 days at 37°C, the contents of the dialysis tubing were centrifuged at 3670 *g* and 4°C for 20 min; the culture supernate was retained and filtered (0.45 µm).

Bovine thyroglobulin affinity chromatography

The method was based on that of Krivan and Wilkins¹² as described previously.¹³ Briefly, 250 mg of bovine thyroglobulin (Sigma) were dissolved in 50 ml of 0.1 M morpholine propane sulphonic acid buffer (pH 7.0), centrifuged (8000 *g*) and filtered (0.2 µm). The thyroglobulin solution was allowed to react with 10 ml of activated Affi-Gel 15 (BioRad Laboratories) overnight at 4°C with shaking. Most (95%) of the thyroglobulin was bound to the beads. After blocking the remaining active sites on the gel with 0.1 M ethanolamine for 30 min at 4°C, the beads were packed into a column (Pharmacia, Uppsala, Sweden; column c 10/10, 10 × 100 mm) which was then washed at 37°C with 20 bed volumes (120 ml) each of pre-warmed basic buffer (0.1 M glycine-sodium hydroxide containing 0.5 M NaCl, pH 10.0) and acidic buffer (0.1 M glycine-hydrochloride containing 0.5 M NaCl, pH 2.0), followed by equilibration at 4°C with 20 bed volumes of 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.0 (TBS). *C. difficile* culture filtrate (100 ml) was applied to the column at 4°C. After washing the column with 25 ml of TBS at 4°C, it was warmed at 37°C for 2 h, and thermal elution was performed by washing with 50 ml of pre-warmed TBS at 37°C. Fractions (5 ml) of the washing at 4°C and the eluates obtained at 37°C were collected and monitored for absorbance at 280 nm (A_{280}), cytotoxicity and haemagglutinating activity as described below.

Anion-exchange chromatography

Two kinds of anion-exchange gel—Q Sepharose FF (Pharmacia) and Mono Q (Pharmacia)—incorporated into a fast protein liquid chromatography (FPLC) apparatus (Pharmacia),¹⁶ were used to obtain highly purified toxin A as reported previously.¹⁴ The sample was filtered through a membrane filter (0.2 µm) before being applied to the column via a 10-ml Super Loop (Pharmacia). Elution was by a 0–0.1 M NaCl gradient in 20 mM Tris-HCl, pH 7.5. Each fraction was examined for A_{280} , cytotoxicity and haemagglutinating activity. The fractions that were both cytotoxic and haemagglutinating (toxin A) after Q Sepharose FF anion-exchange chromatography were dialysed against 20 mM Tris-HCl, pH 7.5, overnight at 4°C before Mono Q anion-exchange chromatography.

Cytotoxicity assay

Cytotoxicity was examined with African green monkey kidney (Vero) cells as described previously.¹³ The cultured cells grown in Eagle's Minimum Essential Medium (MEM) supplemented with calf serum 10% were used for the assay within 1 day. Two- or 10-fold serial dilutions of samples were prepared in PBS. Each diluted sample (100 µl) was added to the cells in a microtitration plate, in the presence of 100 µl of maintenance medium (Eagle's MEM containing calf serum 2%). The cytotoxic titre (cytotoxic unit; CU/100 µl) was expressed as the highest dilution that induced a 100% cytopathic effect (CPE) after incubation for 24 h.

Haemagglutination (HA) assay

This assay¹⁰ was modified by the use of 1.0%, instead of 2.5%, rabbit red blood cells (RBC). Two-fold serial dilutions of samples (50 µl) were made in isotonic buffer (0.1 M Tris, pH 7.2, containing 50 mM NaCl) in V-bottom microtitration plate wells. An equal volume (50 µl) of 1% rabbit RBC washed three times with the isotonic buffer was added to each well. After shaking thoroughly, the plate was incubated at 4°C for 3 h. The HA titre was assessed macroscopically.

Gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli¹⁷ on a 1.5 mm thick 5% resolving slab gel. Samples were diluted 1 in 2 in double strength SDS-PAGE sample buffer (pH 6.8 with 125 mM Tris-HCl-4% SDS, glycerol 20%, with or without β-mercaptoethanol 10%). After boiling the samples for 3 min, electrophoresis was performed with a constant current of 30 mA/gel for 2.5–3 h. High mol. wt markers were purchased from BioRad Laboratories (Richmond, CA, USA).

'Native PAGE' was performed with a 4–30% gradient gel as indicated above for SDS-PAGE, except that SDS and β-mercaptoethanol were left out of the running buffers, gels and samples. The samples for the native gels were not denatured by heating. After pre-electrophoresis at 75 V for 30 min, samples were loaded and electrophoresis was performed at 125 V for 18 h. For estimation of M_r , high M_r standards (Pharmacia) were used. Both SDS-PAGE and native PAGE gels were silver stained as instructed by the manufacturers (BioRad Bulletin 1089), or stained with Coomassie Blue R-250.

Protein assay

Protein concentration was determined by the dye-binding method¹⁸ with the BioRad protein assay kit. Bovine serum albumin was used as a standard.

Immunoblotting

After electrophoresis of denatured and reduced toxin, the samples were blotted^{19,20} on to nitrocellulose

membranes (Schleicher and Schull, Dassel, Germany; BA 83, 0.2 μ m) with BioRad transblot equipment, operated at 30 mA for 12 h and then at 120 mA for 1 h in 0.025 M Tris, 0.192 M glycine containing methanol 13%. The nitrocellulose membranes were rinsed with PBS and blocked for 30 min at room temperature with PBS containing bovine serum albumin 0.18% and Tween 20 0.1%. The membranes were incubated for 3 h at room temperature in either mouse A antitoxin or a mouse monoclonal antibody (MAb PCG-4) to toxin A. The former was obtained from a mouse immunised with highly purified and strongly haemagglutinating toxin A,¹⁴ and the latter was kindly provided by Dr D.M. Lyerly (Virginia Polytechnic Institute and State University). After incubation, the membranes were rinsed three times in PBS and incubated in a 1 in (500–1000) dilution of goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma) for 2 h at room temperature. The membranes were rinsed once with PBS and six times with 40 mM Tris containing 0.16 M NaCl, and blotted proteins were detected with 4-chloro-1-naphthol (Sigma).

To determine whether native toxin A and A' were recognised by MAb PCG-4, they were examined by native-PAGE with the BioRad Mini-Protean II Dual Slab Cell. The toxin A and A' preparations were stacked in an acrylamide 4% stacking gel and separated in an acrylamide 7.5% resolving gel with a Tris-glycine buffer system (25 mM Tris-HCl, 192 mM glycine, pH 8.3). Approximately 0.6 μ g of protein was loaded in each lane and electrophoresed at a constant voltage of 200 V for 1 h. The gel was pre-equilibrated for 30 min in buffer containing 25 mM Tris, 192 mM glycine, pH 8.3, and the proteins were transferred to nitrocellulose at 100 V for 1 h in the same buffer by means of the BioRad Mini Trans-blot Electrophoretic Transfer Cell. After completion of electrophoretic transfer the nitrocellulose membrane was washed (two 5-min washes) in 20 mM Tris, 500 mM NaCl buffer, pH 7.5 (TBS). Non-specific binding was blocked by incubation for 1 h in gelatin 3% w/v in TBS and the membrane was washed (two 5-min washes) in Tween 20 0.05% in TBS (TTBS). The membrane was incubated overnight in MAb PCG-4 diluted 1 in 1000 in gelatin 1% w/v in TTBS, and washed (two 5-min washes) in TTBS. This was followed by incubation for 4 h in rabbit anti-mouse IgG labelled with horseradish peroxidase and diluted 1 in 500 in gelatin 1% w/v in TTBS. The membrane was washed in TTBS (two 5-min washes) and in TBS (one 5-min wash) and developed in a substrate solution prepared by adding 30 mg of 4-chloro-1-naphthol in 10 ml of ice cold methanol to 50 ml of TBS containing H₂O₂ 0.015%.

Dialysis of purified toxin A against BHI broth

In an attempt to show that toxin A' was not simply toxin A to which a soluble receptor from the complex growth medium had attached, purified toxin A in PBS

was dialysed against BHI with the same dialysis bag system as that used for the growth of *C. difficile* and toxin production (see above). The HA and cytotoxicity titres of the toxin A preparation were determined just before dialysis and again after dialysis for 48 h at 37°C anaerobically.

Analysis of toxin A produced by C. difficile in a chemically defined medium

In an additional approach to exclude the possibility that toxin A' was toxin A combined with a soluble medium component that blocked HA receptors, the BHI medium was replaced by a chemically defined medium²¹ which contained nine amino-acids, five mineral salts, nicotinic acid, riboflavin and the monosaccharide N-acetylglucosamine. *C. difficile* was then cultured by the dialysis bag method and the toxins were purified by Q-Sepharose FF and Mono Q anion-exchange chromatography.

Results

Mono Q anion-exchange chromatography profile of C. difficile culture filtrate

To examine the relationship between the cytotoxic and HA activities of toxin A, 0.5 ml of *C. difficile* culture filtrate (protein 1.55 mg/ml, cytotoxicity 6.7×10^7 CU/100 μ l, HA titre 64) was fractionated by Mono Q anion-exchange column chromatography and each fraction (0.25 ml) was examined for cytotoxicity and HA activity (fig. 1). Two peaks of cytotoxicity representing toxins A and B were demonstrated in fractions 35–45 and 65–75, which were eluted at 0.27–0.37 M and 0.57–0.67 M NaCl, respectively. The toxin B fractions showed no HA activity despite having high titres (10^7 – 10^8 CU/100 μ l) of cytotoxicity, whereas in the toxin A fractions a peak titre (128) of HA activity was detected. Unexpectedly, the peaks of cytotoxicity and HA activity of toxin A were not coincident. The highest titre (10^5) of cytotoxicity was detected in fractions 39–40, whereas the HA peak was observed in fraction 41, demonstrating a discordance between peaks of the two activities of toxin A. To highlight this further, a step-wise elution from 0.30 to 0.32 M NaCl was performed by Mono Q-FPLC after loading the same amounts (0.5 ml) of crude culture filtrate (table I); 0.25-ml fractions were collected. In this experiment, the cytotoxicity titre was determined from a two-fold serial dilution to obtain a more exact endpoint. Fraction 2 gave the highest titre (2^{14}) of cytotoxicity and had an HA titre of 16. The highest HA titre (32) was detected in fraction 3, which had a cytotoxicity titre of 2^{13} . More significantly, fraction 1, which also had a cytotoxicity titre of 2^{13} , had no HA activity. As fractions 1–3 eluted at the same concentration of NaCl (0.30 M) the discordance between the peaks of the two biological activities was not due to

Fig. 1. Mono Q-FPLC profile of *C. difficile* culture filtrate (0.5 ml) after elution with 0–1.0 M NaCl; 0.25-ml fractions were collected and examined for cytotoxicity (O) and HA activity (▲). (■) NaCl concentration (M).

Table I. Relationship between cytotoxicity and HA activity of *C. difficile* toxin A fractions separated by FPLC with a Mono Q anion-exchange column

Fraction no.	NaCl (M)	Cytotoxicity (CU/100 μ l)	HA activity (titre)
1	0.30	2^{13}	<1
2	0.30	2^{14}	16
3	0.30	2^{13}	32
4	0.30	2^{10}	16
5	0.31	2^9	4
6	0.31	2^9	2
7	0.31	2^9	1
8	0.31	2^8	1
9	0.32	2^7	1
10	0.32	2^6	<1
11	0.32	2^5	<1
12	0.32	2^5	<1

slight differences in NaCl concentration in the two assays.

Analysis of purified toxin A

A similar experiment was next made with pure toxin A, instead of with crude culture filtrate. The purification procedure consisted of three sequential

chromatographic steps—thyroglobulin affinity (TG) chromatography, Q-Sepharose FF anion-exchange chromatography, and Mono Q anion-exchange chromatography. When loaded on to a Q-Sepharose FF column, the thermally eluted TG toxin A specimen (TG fractions 26–29, 20 ml) eluted maximally at 0.44–0.46 M NaCl (fraction 24) with a cytotoxic titre of 2^{12} and an HA titre of 64. Fractions corresponding to toxin A (fractions 23–25, 6 ml) were loaded on to a Mono Q column and subjected to 0–1.0 M NaCl elution. Small fractions (0.25 ml) were collected and examined for cytotoxicity and HA activity (table II). Discordance was noted between the peaks of the two activities, though it was much less striking than with the culture filtrate. This was to be expected (see below) because non-haemagglutinating toxin A would have been lost as a result of the thyroglobulin affinity purification step.

Analysis of TG chromatography unbound material for non- or weakly haemagglutinating toxin A

The twin peaks of biological activity described above suggested the existence of a variant of toxin A with little or no HA activity. Such a variant would be expected to pass more readily through a TG column,

Table II. Cytotoxicity and HA activity of the fractions separated by FPLC with a Mono Q anion-exchange column from highly purified toxin A or thyroglobulin (TG)-unbound toxin A preparation

Fraction no.	NaCl (M)	Highly purified toxin A		TG-unbound toxin A	
		Cytotoxicity (CU/100 μ l)	HA activity (titre)	Cytotoxicity (CU/100 μ l)	HA activity (titre)
36	0.28	< 2^3	<2	< 2^3	<2
37	0.29	< 2^3	<2	2^6	<2
38	0.30	2^{11}	4	2^{13}	<2
39	0.31	2^{13}	32	2^{14}	<2
40	0.32	2^{12}	64	2^{13}	2
41	0.33	2^{10}	16	2^{13}	2
42	0.34	2^8	4	2^{11}	<2
43	0.35	2^6	<2	2^{10}	<2

as the receptors responsible for HA and interaction with bovine thyroglobulin are thought to be the same.¹² Therefore, thyroglobulin unbound fractions (TG fractions 10–13, 20 ml), which contained toxin B and unknown proteins, were also analysed by Q-Sepharose FF anion-exchange column chromatography. A fraction (fraction 24) eluting at 0.44–0.46 M NaCl, the point at which toxin A eluted, had a cytotoxic titre of 2^{13} but an HA titre of only 2. This confirmed that the HA activity did not always correspond to the degree of cytotoxicity of toxin A.

This variant (A') of toxin A was further purified by subjecting Q-Sepharose FF fractions 23–25 (6 ml) to Mono Q anion-exchange column chromatography. Each 0.25-ml fraction was collected and examined for cytotoxicity and HA activity (table II). The highest titre of cytotoxicity (2^{14}) was detected in fraction 39, eluting at 0.31 M NaCl. Interestingly, this fraction had no detectable HA titre. In fractions 40–41, an HA titre of only 2 was detected. These results give further confirmation of the existence of a variant of toxin A.

Effect of growth in a chemically defined medium and of dialysis of toxin A against BHI broth on concentration of toxin A'

The dialysis of a purified toxin A preparation against BHI had no effect on the cytotoxicity or HA titres, which remained at 2^{13} and 64 respectively. Toxin A/A', produced by growth in a chemically defined medium and purified by Q-Sepharose FF and Mono Q anion-exchange chromatography, yielded seven 1-ml fractions which eluted where toxin A/A' would be expected. The cytotoxic titre of these fractions ranged from 2^8 to 2^{14} , but none had an HA titre.

Polyacrylamide gel electrophoresis (PAGE)

Equal volumes (40 μ l) of highly purified toxin A and Q-Sepharose FF-Mono Q-separated toxin A' (fraction 39) were compared by native and SDS-PAGE (figs. 2–4). After native gel electrophoresis, only one band (540 Kda) of toxin A was detected by silver stain. In contrast, TG-unbound Q-Sepharose FF-Mono Q-separated toxin A' exhibited an additional six faint bands (490, 430, 230, 130, 105 and 40 Kda).

The SDS-PAGE profiles of toxins A and A' (fraction 39), both with and without reduction with β -mercaptoethanol, are shown in figs. 3 and 4. In non-reducing conditions, one major band (a doublet) of 240 Kda, nine minor bands and many faint bands were detected from highly purified toxin A (fig. 3). Although the same profile was obtained from toxin A' an additional five bands smaller than 45 Kda were also detected (fig. 3).

Analysis of these toxin A and A' fractions by SDS-PAGE in reducing conditions (fig. 4) showed that, in addition to the major band of 240 Kda, there were numerous bands common to both preparations, and

several bands (<45 Kda) apparently unique to the toxin A' preparation.

Immunoblotting of toxins A and A'

As toxins A and A' have similar physico-chemical properties (mol. wt; breakdown profile after denaturation with or without reduction; and charge based on point of elution after anion-exchange chromatography) it was decided to investigate their antigenic similarities. Immunoblotting with mouse mono-specific A antitoxin showed some differences between toxins A and A' (fig. 5A), but the major 240-Kda peptide was strongly developed in both toxin preparations. In non-reducing conditions (lanes 1 and 2), five minor bands of >240 Kda were developed more strongly in toxin A than in A'. Similarly, five faint bands (96, 86, 70, 60 and 38 Kda) were apparent in toxin A but not in A' (not seen on photographic reproduction). In reducing conditions (lanes 3 and 4),

Fig. 2. Native PAGE of Mono Q-FPLC-separated toxin A and toxin A' fractions. Thyroglobulin affinity-Q-Sepharose FF-separated toxin A (20 ml) or thyroglobulin affinity unbound Q-Sepharose FF-separated toxin A' (20 ml) was further purified by Mono Q-FPLC; 0.25-ml fractions were collected after 0.1–0 M NaCl elution. Peak fraction 39 (40 μ l) of either toxin A or A' was loaded on to a 4–30% gradient gel without SDS. After electrophoresis (125 V, 18 h), the gel was developed with silver. Centre lane: mol. wt standards—thyroglobulin (669 Kda), ferritin (440 Kda), catalase (232 Kda), lactate dehydrogenase (140 Kda), bovine serum albumin (67 Kda) (Pharmacia).

Fig. 3. SDS-PAGE in non-reducing conditions of Mono Q-FPLC-separated toxin A and toxin A' fractions. From each peak fraction eluted from the Mono Q column (fraction 39 for both toxin preparations), 40 μ l were mixed with an equal volume of sample buffer without β -mercaptoethanol, and boiled for 3 min. After electrophoresis (30 mA, 2.5–3 h), the gel was developed by silver. First lane: mol. wt standards—myosin (200 Kda), β -galactosidase (116 Kda), phosphorylase B (92.5 Kda), bovine serum albumin (66 Kda; BioRad), ovalbumin (45 Kda). *Doublet at 240 Kda.

three further bands (175, 160 and 155 Kda) were more intensely developed in toxin A than in A', and the faint bands at 96 (not seen on photographic reproduction), 86, 70, 60 and 38 Kda were more apparent (lane 3).

In contrast, immunoblotting with MAb PCG-4 gave patterns (figs. 5B and 6) different from those produced by mono-specific A antitoxin. In non-reducing conditions, five bands larger than the 240-Kda doublet developed to a similar extent in both toxins A and A' (fig. 5B; lanes 1 and 2). In reducing conditions, many bands developed, in addition to the major 240-Kda band in toxin A' (fig. 5B; lane 4). In contrast, toxin A exhibited three clear bands: the major one of 240 Kda, and two bands of 160 and 155 Kda (fig. 5B; lane 3). The 160-Kda band was more

strongly developed in toxin A than in A', the opposite being true for the 155 Kda band. In both reducing and non-reducing conditions, four bands (195, 180, 175 and 125 Kda) were detected only in toxin A' (fig. 5B; lanes 2 and 4). Both native toxin A and toxin A' were recognised by MAb PCG-4 in immunoblots after transfer of the toxins from a native gel (fig. 6). An additional minor band was recognised in toxin A'.

Discussion

At least two toxins, toxin A (enterotoxin) and toxin B (cytotoxin) are produced by *C. difficile*. Some investigators have reported an additional enterotoxin which is unstable and induces fluid accumulation without haemorrhage in rabbit ligated ileal loops,^{5,22} but these findings need to be confirmed. Among these

Fig. 4. SDS-PAGE in reducing conditions of Mono Q-separated toxin A and A' fractions. For each toxin preparation, 40 μ l of peak fraction 39 eluted from the Mono Q column were mixed with an equal volume of sample buffer with β -mercaptoethanol 5%. First lane, mol. wt markers (see fig. 3). *Major 240 Kda component.

Fig. 5. Immunoblot analysis of toxins A and A'. Highly purified toxin A (7 μ g) (lanes 1 and 3) and Mono Q separated toxin A' (7 μ g) (2 and 4) were loaded on to a 5% gel and SDS-PAGE was performed in non-reducing (lanes 1 and 2) or reducing (3 and 4) conditions. (A) Mouse monospecific anti-toxin A (diluted 1 in 500) was used for reaction with the transferred proteins. (B) Mouse MAb to toxin A (PCG-4; diluted 1 in 500) was used for reaction with the transferred proteins. *Major 240 Kda component.

toxins, A has been regarded as the most important virulence factor,^{23,24} because it—but not toxin B—induces fluid accumulation with haemorrhage in the ligated intestinal loops of rabbits.^{5,25} The enterotoxicity of toxin A has also been shown in infant or suckling mice⁶ and golden Syrian hamsters.²⁶

Toxin A binds to brush border membranes of hamsters, indicating the presence of a receptor, and to rabbit erythrocytes, which it agglutinates at 4°C but not 37°C.¹⁰ These observations led to the development of an efficient affinity procedure for purifying toxin A, based on the presence of the trisaccharide receptor on bovine thyroglobulin.¹² The method is improved by subsequent anion-exchange chromatography.¹³ Analysis of culture filtrate by Mono Q anion-exchange chromatography showed that the peaks of HA and cytotoxicity in culture filtrate did not coincide; neither did they in purified toxin A obtained by the modified thyroglobulin affinity chromatography method¹³ though the discrepancy was less striking. These results suggest the existence of toxin A molecules with

Fig. 6. Immunoblot analysis of toxins A and A' with MAb PCG-4 after transfer from a polyacrylamide 7.5% native gel. Lane 1: highly purified toxin A (0.6 μ g) and lane 2: toxin A' (0.6 μ g).

differing HA properties, which may explain a reported micro-heterogeneity of toxin A.²⁷ Poor or non-haemagglutinating forms, if present, would more readily pass through the thyroglobulin affinity column at 4°C. Analysis of thyroglobulin unbound material, which contained toxin B and many other proteins, demonstrated toxin A with full cytotoxicity but negligible HA properties. This preparation, toxin A', could be partly purified by Q Sepharose FF anion-exchange chromatography and further purified by Mono Q anion-exchange chromatography.

The final toxin A' preparation contained a few minor contaminant proteins in addition to a major 540-Kda protein, but the relatively pure material from Mono Q fractions 38–40 (eluted at 0.30–0.32 M NaCl) had a low HA titre (2 or less) but was highly cytotoxic (2¹³–2¹⁴). That this was not toxin A denatured during thyroglobulin chromatography, leaving the binding components associated with the column, was evident from the fact that weakly haemagglutinating but potentially cytotoxic forms of toxin A were detected even on direct anion-exchange analysis of crude culture filtrate.

It is unlikely that toxin A' was simply the result of interaction of toxin A with BHI components that blocked the Gal α 1-3Gal β 1-4GlcNAc receptors on the toxin molecule and thereby prevented HA. Firstly, dialysis of purified toxin A against BHI broth did not result in any loss of HA activity; had soluble BHI components interacted with toxin A, some such loss would have been expected. Secondly, growth in a chemically defined medium yielded predominantly toxin A'; it also leads to a diminished production of proteases,²⁸ which may play a part in the conversion of toxin A' to toxin A.

Denaturation of toxin A by SDS, with or without β -mercaptoethanol, was shown previously to cause dissociation into a major 240-Kda protein (appearing as a doublet) and 10 minor and 27 faint bands (non-reduced), and a major 240-Kda protein and four minor and 31 faint bands (reduced).¹⁴ A similar dissociation has been shown by others.^{27,29} Highly purified toxin A and Q Sepharose-FF-Mono Q-separated toxin A' were similar but not identical. It proved impossible to elute cytotoxicity and HA activity from the SDS-PAGE gels in order to examine the bands associated specifically with either activity.

Even though toxin A' exhibited no HA activity, it was recognised by MAb PCG-4 in immunoblots of its denatured non-reduced and denatured reduced forms in a pattern similar to that of toxin A.

This observation indicated that toxin A' differed from toxin A configurationally, the HA components being masked in the native molecule. However, both toxins A and A' were readily recognised in an enzyme immunoassay (unpublished data) and in an immunoblot of native toxin. This may indicate that MAb PCG-4 recognises an epitope different from that responsible for binding and prevents HA as a result of steric hindrance, or that the relevant epitope in toxin

A' may be accessible to MAb PCG-4 but not to the receptor on rabbit erythrocytes.

Because toxin A' possessed cytotoxic but not HA activity, the receptors associated with these two functions (and, by analogy, binding to brush-border membranes and enterotoxicity) would appear to be different. This accords with the observation that PCG-4 neutralises the enterotoxic but not cytotoxic activity of toxin A.^{24,29} On the other hand, mouse embryonal carcinoma cells that produce high amounts of the trisaccharide Gal α 1-3Gal β 1-4GlcNAc are much more sensitive to the cytotoxic activity of toxin A than are

those that do not.³⁰ It seems possible that toxin A uses a variety of different cell receptors for the initial binding step to cells in tissue culture, and that the Gal α 1-3Gal β 1-4GlcNAc trisaccharide is simply one of the preferred ones.

The evidence presented strongly indicates that there is a form of toxin A (probably a pro-toxin) that has a weak ability to haemagglutinate rabbit erythrocytes but is potentially cytotoxic.

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