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Environnement Canada

Mucosal immunization with Clostridium botulinum type C 16 S toxoid and its non-toxic component

By:
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ABSTRACT

Mucosal innumisation with Clostridium botulinum type C16 S toxoid and its non-toxic component.

Clostridium botulinum types C and D produce a 16 S (500 kDa)toxin that is formed by conjugation of neurotoxin with a non-toxin component (non Tox). The amino acid sequences od type C and D nonTox components are almost identical. In a previous report it was proposed that nonTox is necessary for the effective absorption of the toxin from the small intestine. This suggested the hypothesis that mucosal immunity against nonTox in the small intestine might prevent the absorption of both C- and D-16 S toxins. The nonTox was purified from a mutant strain, (C)-N71, that does not produce neurotoxin. This nonTox or detoxified C-16 toxin were mixed with adjuvant (a mutant form of heat labile toxin of *Escherichia coli*), and inoculated nasally four times with nonTox or toxoid produced high levels of antibodies. (including IgA) against the immunogens, both in intestinal fluids and sera. When these nonTox-immunised mice were challenged orally with 2 and 20 oral minimum lethal doses (MLD) of C- or D-16 S toxins, the same results were obtained with both C and D; the mice survived after challenge with 2 MLD of either C or D but were killed by 20 MLD of either toxin although the time to death was significantly longer than in the control non-immunised mice. These results indicate that the local anti-nonTox antibodies reduce absorption of both C- and D-16 toxins from the small intestine. The C-16 S toxoid-immunised mice showed similar behaviour with type D toxin challenge, probably due to the same mechanism, but were protected against 20 MLD of C-16 S toxin.

NWRI RESEARCH SUMMARY

Plain language title
A vaccine for type C botulism

What is the problem and what do sicentists already know about it?
Botulism kills as many waterfowl as are reared in all habitant restoration programs. At times, farm animals die. Rarely people also die from botulism but not from the type of botulism in this study. The ability to control this disease in wildlife varies from difficult to impossible. Livestock can be protected with vaccines.

Why did NWRI do this study?
Management of outbreaks of avian botulism is still a serious concern. Vaccines are not considered a general tool for treatment of wildlife but an effective vaccine may provide protection for special genetic stocks or for research purposes. NWRI has been collaborating with Okayama University on botulism management for a number of years.

What were the results?
A new vaccine for type C botulism was developed. It can provide protection from typical exposures to type C botulism found in nature.

How will these results be used?
The primary use of this vaccine will likely be for domesticated animals. For example, in Australia, horses and cows often chew on bones of dead animals. They are attempting to get salt but without treatment can die of botulism.

Who were our main partners in the study?
The School of Medicine and Dentisty, Okayama University was the primary agency. Other team members were: James Cook University, Australia; Fujita Health University, Japan; Tokyo University of Agriculture, and Kanazawa University, Japan

Publishing Information
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RÉSUMÉ

Immunisation mucosale avec l'anatoxine 16S de *Clostridium botulinum* de type C et son constituant non toxique.

Les types C et D de *Clostridium botulinum* produisent une toxine 16S (500 kDa) formée par conjugaison d'une neurotoxine à un constituant non toxique. Les séquences d'acides aminés du constituant non toxique du type C et du type D sont presque identiques. Dans un rapport précédent, on a indiqué que le constituant non toxique est nécessaire pour une absorption efficace de la toxine dans l'intestin grêle. Cela laissait supposer que l'immunité mucosale contre le constituant non toxique dans l'intestin grêle peut empêcher l'absorption de la toxine 16 S des types C et D. Le constituant non toxique a été purifié à partir d'une souche mutante, (C)-N71, qui ne produit pas de neurotoxine. Ce constituant non toxique ou la toxine C-16 détoxifiée ont été mélangées à un adjuvant (une forme mutante de la toxine thermolabile de *Escherichia coli*), et des souris ont été inoculées quatre fois par voie intranasale avec le constituant non toxique ou l'anatoxine de manière à produire des taux élevés d'anticorps (y compris des IgA) contre les immunogènes, dans le liquide intestinal et le sérum. Lorsque ces souris immunisées par le constituant non toxique ont été soumises par voie orale à 2 et 20 doses létales minimales (DLM) de toxine des types C ou D, les mêmes résultats ont été obtenus pour les types C et D. Les souris ont survécu à la dose de 2 DLM de toxine des types C et D, mais ont été tuées par la dose de 20 DLM des deux toxines, quoique elles aient survécu significativement plus longtemps que les souris témoins non immunisées. Ces résultats indiquent que les anticorps intestinaux dirigés contre le constituant non toxique réduisent l'absorption des toxines des types C et D dans l'intestin grêle. Les souris immunisées avec l'anatoxine C-16S se sont comportées de la même façon face à la toxine de type D, probablement à cause du même mécanisme, mais elles étaient protégées contre 20 DLM de la toxine C-16S.

Sommaire des recherches de l'INRE

Titre en langage clair
Vaccin contre le botulisme de type C

Quel est le problème et que savent les chercheurs à ce sujet?
Le botulisme tue autant de sauvagine qu'on en réintroduit dans tous les programmes de restauration de l'habitat. Cette maladie tue parfois des animaux d'élevage. Il arrive également, quoique rarement, que des gens meurent du botulisme, mais il ne s'agit pas du type de botulisme étudié ici. La lutte contre cette maladie dans les populations sauvages est difficile, voire impossible. Le bétail peut être protégé par des vaccins.

Pourquoi l'INRE a-t-il effectué cette étude?
La gestion des flambées de botulisme aviaire est source de graves préoccupations. Les vaccins ne sont pas considérés comme des outils d'usage général chez la faune, mais on peut utiliser un vaccin pour conférer une protection efficace à des géniteurs spéciaux ou encore à des fins de recherche. L'INRE collabore depuis un certain nombre d'années avec l'Université d'Okayama sur la gestion du botulisme.

Quels sont les résultats?
Un nouveau vaccin a été mis au point contre le botulisme de type C. Ce vaccin offre une protection efficace contre les expositions habituelles au botulisme de type C dans la nature.

Comment ces résultats seront-ils utilisés?
Ce vaccin sera probablement utilisé surtout chez les animaux domestiques. Par exemple, en Australie, des chevaux et des vaches peuvent contracter le botulisme en mâchant des os d'animaux morts, à la recherche de sel. Sans traitement, ces animaux peuvent mourir.

Quels étaient nos principaux partenaires dans cette étude?
Le principal organisme participant est la Faculté de médecine et de dentisterie de l'Université d'Okayama. Les autres membres de l'équipe sont : l'Université James Cook d'Australie, l'Université de la santé Fujita du Japon, l'Université agricole de Tokyo et l'Université de Kanazawa du Japon.

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HOST RESPONSE TO INFECTION

Mucosal immunisation with *Clostridium botulinum* type C 16 S toxoid and its non-toxic component

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***Clostridium botulinum* types C and D produce a 16 S (500 kDa) toxin that is formed by conjugation of neurotoxin with a non-toxic component (nonTox). The amino acid sequences of type C and D nonTox components are almost identical. In a previous report it was proposed that nonTox is necessary for the effective absorption of the toxin from the small intestine. This suggested the hypothesis that mucosal immunity against nonTox in the small intestine might prevent the absorption of both C- and D-16 S toxins. The nonTox was purified from a mutant strain, (C)-N71, that does not produce neurotoxin. This nonTox or detoxified C-16 S toxin were mixed with adjuvant (a mutant form of heat-labile toxin of *Escherichia coli*), and inoculated into mice *via* the nasal or oral route, or both. The mice inoculated nasally four times with nonTox or toxoid produced high levels of antibodies (including IgA) against the immunogens, both in intestinal fluids and sera. When these nonTox-immunised mice were challenged orally with 2 and 20 oral minimum lethal doses (MLD) of C- or D-16 S toxins, the same results were obtained with both C and D; the mice survived after challenge with 2 MLD of either C or D but were killed by 20 MLD of either toxin although the time to death was significantly longer than in the control non-immunised mice. These results indicate that the local anti-nonTox antibodies reduce absorption of both C- and D-16 S toxins from the small intestine. The C-16 S toxoid-immunised mice showed similar behaviour with type D toxin challenge, probably due to the same mechanism, but were protected against 20 MLD of C-16 S toxin.**

Introduction

Clostridium botulinum strains produce seven immunologically distinct neurotoxins, types A–G. The neurotoxins inhibit the release of acetylcholine at the neuromuscular junctions and synapses and cause botulism in man and animals. All types of neurotoxin are synthesised as a single chain with Mr of *c.* 150 kDa. Endogenous protease(s) from bacteria or exogenous proteases such as trypsin cleave the single chain neurotoxins at about one third of the length from the N-terminus within a region inside a disulphide loop. Therefore, neurotoxins become dichains consisting of 50-kDa (designated light chain, L) and 100-kDa (heavy chain, H) components held together by a disulphide bond.

The neurotoxins are associated with a non-toxic (nonTox) component in cultures or in foods, and become large complexes designated progenitor toxins. Type C and D strains produce two forms of progenitor toxin, 12 S (300 kDa, M toxin) and 16 S (500 kDa, L toxin). The 12 S toxin consists of a neurotoxin (7 S)

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and a nonTox showing no haemagglutinin (HA) activity, which is described here as non-toxic non-HA. The Mr of non-toxic non-HA is similar to that of the neurotoxin. The 16 S toxin is formed by conjugation of M toxin with the other nonTox named as haemagglutinin (HA). Previous studies found that HA consists of four subcomponents designated HA1 (33 kDa), HA2 (17 kDa), HA3a (22–23 kDa) and HA3b (53 kDa), and that the non-toxic non-HA of 12S toxin and HA3a are cleaved at their N-terminal regions [1–5]. Homology of amino acid sequences of C and D neurotoxins is *c.* 60%, but that of nonTox components is almost identical [6–8]. Human and animal food-borne botulism is caused by ingestion of food or feed containing the progenitor toxin. The progenitor toxins dissociate into neurotoxins and nonTox components under alkaline conditions; 12 S toxin dissociates into neurotoxin and non-toxic non-HA, and 16 S toxin dissociates into neurotoxin and nonTox which is a complex of non-toxic non-HA and HA. Sakaguchi and co-workers concluded that the nonTox is necessary to cause food-borne botulism because the nonTox protects the neurotoxin from the acidity and proteases in the digestive tract. They found that larger sized toxin has greater stability and, therefore, possesses higher oral toxicity [9, 10]. Furthermore, they proposed that the progenitor toxin dissociates into neurotoxin and nonTox in the lymphatic system after absorption from the small intestine as the dissociation was not observed under the alkaline conditions of intestinal juice, and that the absorption rates of neurotoxin, 12 S and 16 S toxins are similar based on the data obtained by injecting these toxins into the ligated rat small intestine [11]. Maksymowych *et al.* [12] reported that nonTox had no effect on the absorption of the toxins, and that the neurotoxin alone could be absorbed from the stomach and small intestine in mice.

Similar absorption experiments were performed in this laboratory with purified type C neurotoxin, 12 S and 16 S toxins in guinea-pigs. Because it was speculated that ligation may cause tissue damage, these toxins were inoculated into the small intestine without ligation. Only the 16 S toxin bound obviously to the epithelial cells and time to death after injection with 16 S toxin was measurably shorter compared with 12 S toxin or neurotoxin [13]. Furthermore, in a way comparable to 16 S toxin, recombinant proteins of HA1 and HA3 could bind both epithelial cells and erythrocytes [14, 15]. Some monoclonal antibodies against HA1 reduced the 16 S toxin binding to the epithelial cells and neutralised the oral toxicity of small amounts of 16 S toxin [16]. This suggested the hypothesis that HA plays an important role for binding of 16 S toxin to the epithelial cells of the small intestine, leading to effective absorption.

The aim of the work presented here was to attempt to elicit local (intestinal) mucosal immunity against nonTox in mice and to examine whether the local immunity could protect mice from an oral challenge with both type C- and D-16 S toxins. Local immunity was elicited by inoculation into the nasal cavity of a mixture of antigen and a mutant form of heat-labile toxin (LT) of *Escherichia coli* that has high adjuvant activity but little toxigenicity.

Materials and methods

Strains

Strains of C-Stockholm and D-CB16 and mutant strain (C)-N71 were employed. (C)-N71 was previously obtained by treating C-Stockholm with N-methyl-N'-nitro-N-nitrosoguanidine, and is a non-toxic but HA-positive mutant strain [17, 18]. Later, Hauser *et al.* [19] determined that this strain has a stop codon in the gene coding for the L chain of neurotoxin. However, in our experience, nonTox alone was purified from this strain and thus avoided any contamination with neurotoxin.

Toxin, nonTox and toxoid

Type C-16 S toxin and the nonTox were purified by a similar procedure from C-Stockholm and (C)-N71, respectively. The strains were cultured at 35°C for 5 days by a cellophane-tube procedure as reported previously [4, 5]. The culture fluid containing the 12 S and 16 S toxins was centrifuged (*c.* 15 000 *g* for 20–30 min at 20°C; this temperature was maintained throughout the purification procedure except dialysis. Dialysis was always performed at 4°C). The supernate was then fractionated with a 60% w/v saturation of $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, resuspended in 0.05 M sodium acetate buffer (pH 4.2) in about one tenth volume of the starting culture fluid, and then dialysed overnight against the same buffer. Any precipitate that appeared was removed by centrifugation. The supernate was then applied to an SP-Toyopearl 650M (Tosoh, Japan) column equilibrated with 0.05 M sodium acetate buffer (pH 4.2). After elution with an exponential gradient of NaCl (0–0.8 M) in the same buffer, the fractions containing 12 S and 16 S toxins were collected separately. The 16 S toxin fractions were contaminated with a small amount of C3 enzyme. This was eliminated by using a hydroxyapatite (Seikagaku Kogyo, Japan) column equilibrated with 0.01 M sodium phosphate buffer (PB, pH 6.0). The 16 S toxin preparation was dialysed against PB, layered on the column, washed first with PB with 1 M NaCl to elute C3 enzyme, followed by 0.2 M PB (pH 6.0) to elute 16 S toxin.

The nonTox was precipitated from the culture supernate of (C)-N71 with $(NH_4)_2SO_4$ 60% w/v, resuspended, and then dialysed against 0.01 M PB (pH 6.0). The precipitate that appeared during dialysis contained predominately nonTox and free HA-1 (and C3 enzyme), and was collected by centrifugation and

resuspended in 0.05 M sodium acetate buffer (pH 4.2). Thereafter, the nonTox was purified by successive column chromatography with SP-Toyopearl 650S and hydroxyapatite under the same conditions as used for obtaining16 S toxin.

Type D-16 S toxin was purified from strain D-CB16. Incubation and precipitation with $(NH_4)_2SO_4$ 60% w/v were as described for type C. In this case, 16 S toxin, C3 enzyme, and free HA1 appeared in the insoluble fraction during dialysis of the $(NH_4)_2SO_4$-precipitate against 0.02 M PB (pH 6.0) similar to nonTox described above. The precipitate was collected, resuspended in 0.15 M sodium acetate buffer (pH 4.2), and then separated by applying successively to SP-Toyopearl 650 M and Toyopearl HW-65S columns.

The C-16 S toxin was detoxified (toxoided) by dialysis against 0.01 M PB (pH 6.0) containing formalin 0.4% at room temperature for about 10 days.

Adjuvant

A mutant form of LT of *E. coli*, with a deletion of the Arg192–Ile194 region of its A subunit was prepared previously [20]. This preparation showed little toxicity in mice but still had high adjuvant activity for local immunity.

Immunisation schedule and challenge

A portion (1 mg/ml) of type C nonTox or toxoid was mixed with an equal amount (protein and volume) of the adjuvant, and the mixture was inoculated into the nasal cavity (20 μl) or stomach (400 μl, inoculated by a metal probe) of each mouse. Six groups consisting of 10 or 12 mice each were prepared, and were immunised 1–4 times at 1-week intervals according to the protocol shown in Table 1; with toxoid four times nasally (toxoid/N4) or with nonTox (1) four times nasally (nonTox/N4), (2) two times nasally (nonTox/N2), (3) once nasally (nonTox/N1), (4) once nasally followed by three times orally (nonTox/N1-O3) and (5) four times orally (nonTox/O4). Two weeks after the final immunisation, two mice were killed and the sera and intestinal fluids were obtained to check the level of antibody production as described below. The remaining mice were challenged orally with 0.4 ml of either C- or D-16 S toxin in PB, pH 6.0, containing 2 or 20 times the minimum oral lethal dose (oral MLD); toxin inoculation was by a metal probe into the stomach. One oral MLD was determined as the highest dilution of toxin that kills a mouse within 1 day. One oral MLD of type C and D 16 S toxins correspond to *c.* 1.2×10^4 and 4×10^3 intraperitoneal (i.p.) MLD, respectively (i.e., oral inoculation of 0.4 ml of the preparations contained 3×10^4 and 1×10^4 i.p MLD/ml, respectively). The challenged mice were observed for 1 week.

Characterisation of antibodies by ELISA, Western blot and neutralisation test

The small intestines obtained from the dead mice were cut into small pieces in 2 ml of 0.01 M phosphate-buffered saline (PBS, pH 7.4), washed with vigorous shaking, and then centrifuged at low speed. The supernates were used as the intestinal fluids. Intestinal fluids from two mice belonging to the same group were pooled, as were the sera. The IgA and total immunoglobulin (Ig) activities of these intestinal fluids and sera against nonTox, C- or D-16 S toxin were assayed by ELISA, Western blot and toxin neutralisation test.

ELISA, SDS-PAGE and Western blot analysis

ELISA, SDS-PAGE and Western blots were performed as reported previously [16]. For ELISA, preparation of intestinal fluids or sera diluted in skin milk-PRS were added to wells of a microtitration plate coated with 0.5 μg of nonTox or toxin. The serum antibody was peroxidase conjugated-rabbit Igs against mouse Igs or IgA (DAKO, Copenhagen, Denmark) dilution 1 in 1000. For, Western blots, proteins separated by SDS-PAGE were transferrerd to PVDF membrants (ProBlott, Applied Biosystems, Tokyo, Japan). They were exposed to serum diluted 1 in 1000 in skin milk PRS (1 h) and then incubated with peroxidase labelled rabbit Igs against mouse Igs (1 h).

Table 1. Effect of vaccination against C and D toxin challenge

		Mouse lethality after oral challenge with toxin			
		C		D	
Antigen	Schedule (inoculation times)	2 MLD	20 MLD	2 MLD	20 MLD
Toxoid	N4	sss	sss	ss	**dd**
nonTox	N4	sss	**ddd**	ss	**dd**
nonTox	N2	**ssd**	**ddd**	**sd**	ND
nonTox	N1	ddd	ddd	dd	ND
nonTox	N1-03	**ssd**	**ddd**	ss	ND
nonTox	04	ddd	ddd	dd	...
Control	...	ddd	ND	ND	...

N, nasal inoculation; O, oral inoculation; s, survived; d, died as control mice; **d**, died with delayed time compared with control mice; ND, not done.

Toxin neutralisation test. Toxin neutralising activities of the intestinal fluids and sera were investigated. These preparations were serially diluted in 10-fold steps with 0.01 M PBS (pH 7.4) and then mixed with an equal volume of 10 i.p MLD of type C- or D-16 S toxin in 0.01 M PB (pH 6.0). After incubation for 1 h at 37°C in a water bath, the mixtures (0.5 ml) were injected i.p into two mice. The mice were observed for 1 week.

Results

Purification of nonTox from (C)-N71

The culture of (C)-N71 was non-toxic but HA positive, as expected. As described in *Materials and methods*, nonTox precipitated during dialysis of the $(NH_4)_2SO_4$-precipitate of culture supernate against 0.01 M PB (pH 6.0), as did the D-16 S toxin but not the C-16 S toxin. With SP-Toyopearl 650S chromatography, two peaks were obtained. Based on the banding profiles and their N-terminal amino acid sequences, it was clear that the first peak was freely existing HA-1 and the second large peak was nonTox slightly contaminated with C3 enzyme (Fig. 1). Contaminating C3 enzyme was eliminated by the hydroxyapatite column. No neurotoxin-derived band was detected. The purified preparation was again analysed by SDS-PAGE and the N-terminal amino acid sequence of each visualised band was determined (Fig. 2). All subunits of nonTox (non-toxic non-HA, HA1, HA2, HA3a and HA3b) were identified. Three different N-terminal acid sequences were obtained from HA3a, and two clear non-toxic non-HA bands that had the same N-terminal amino acid sequences were detected.

Challenge of immunised mice with toxins

The immunised mice were challenged orally with type C- or D-16 S toxin (Table 1). The mice belonging to the toxoid/N4 group survived challenge with 2 and 20 oral MLD of type C toxin. In the challenge with type D toxin, they were protected against 2 MLD but were killed with 20 MLD, although the time to death was longer than in non-immunised mice; the immunised mice died after 2–3 days. In contrast, the mice of the nonTox/N4 group showed similar results against the challenge with both C and D toxins; they were resistant to 2 MLD, but did not survive 20 MLD of either C or D but, again, the time to death was longer than in control mice. Similar vaccination effects were observed in mice of nonTox/N1-O3 and nonTox/N2 groups, whereas the mice of nonTox/N1 and nonTox/O4 groups

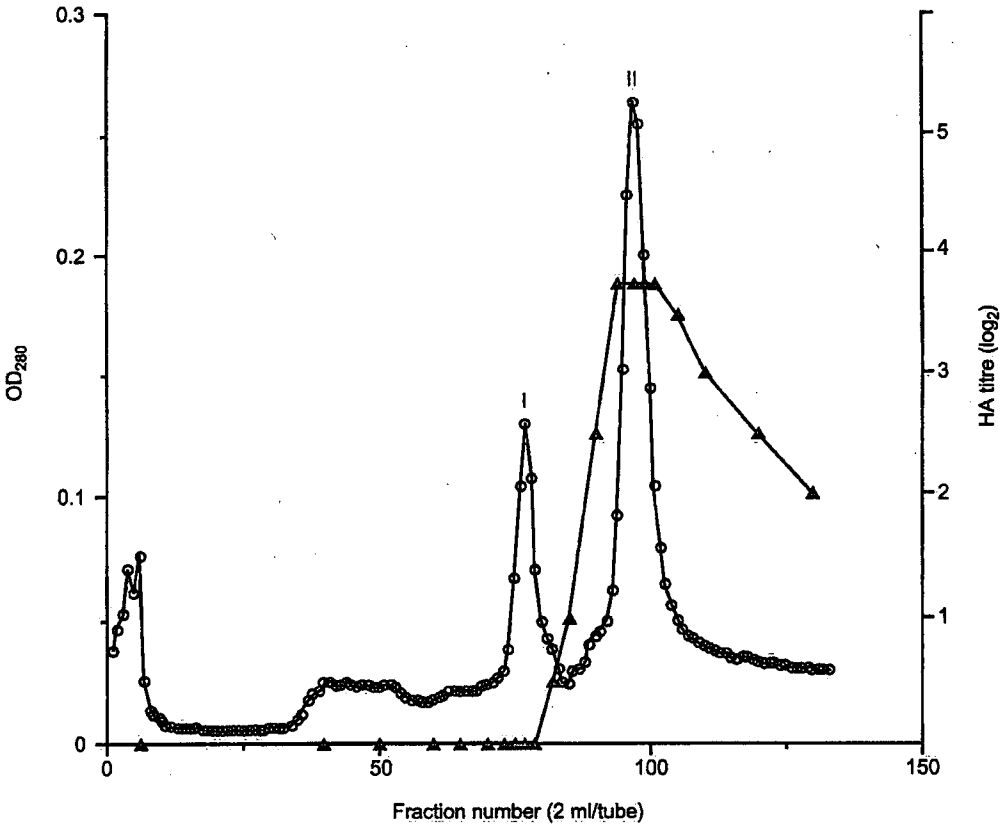

Fig. 1. Elution profile of nonTox of (C)-N71. The $(NH_4)_2SO_4$-precipitate of culture supernate was dialysed against PB. The precipitate that appeared was applied to an SP-Toyopear 650S ion exchange column, and eluted by NaCl gradient with 2 ml per tube. HA titres were determined as reported previously [4]. O, OD_{280}; ▲, HA titre.

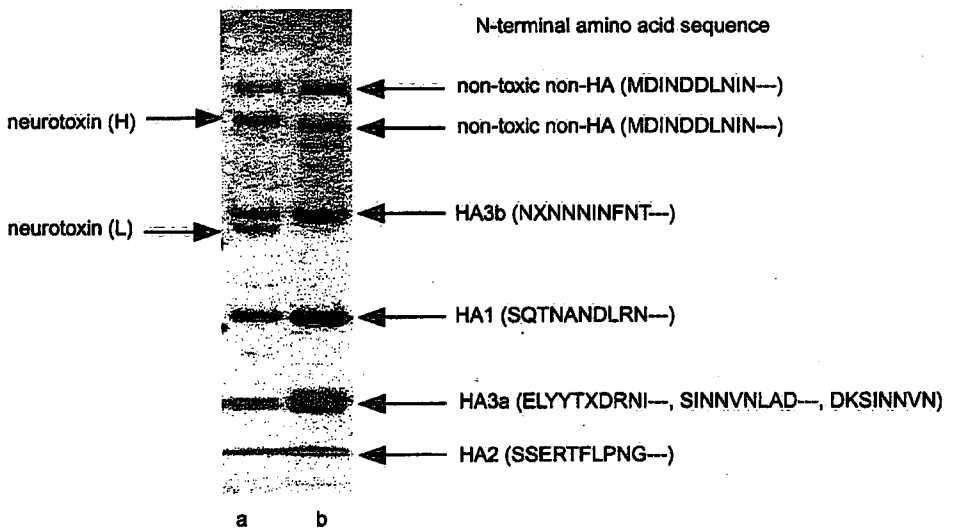

Fig. 2. SDS-PAGE with C-ST 16 S toxin and (C)-N71 nonTox. The purified C-ST 16 S toxin and (C)-N71 nonTox were analysed by SDS-PAGE and the N-terminal amino acid sequence of each band was determined. (a) C-ST 16 S toxin; (b) (C)-N71 nonTox.

did not even survive challenge with 2 MLD of type C toxin.

Antibody production in intestinal fluids and sera

Anti-nonTox IgA and total Ig titres of both intestinal fluids and sera were analysed by ELISA. The mice that survived challenge with 2 MLD of toxin produced high levels of IgA and total Igs in both sera and intestinal fluids, whereas the mice that were killed by the toxin challenge produced only low levels of antibodies (Fig. 3). In all cases, the antibody titres of intestinal fluids were significantly lower than those of sera, probably because of the poor extraction of antibodies from the mucosal tissues and their dilution with the buffer before ELISA (the bits of intestine were shaken up in 2 ml of buffer). Similar reaction profiles were obtained when C- or D-16 S toxin was used instead of nonTox as antigen. The reaction of intestinal fluids and sera from mice of the toxoid/N4 group with C- or D-16 S toxin is shown in Fig. 4.

Reaction of the sera with C-16 S toxin was also analysed by Western blot by employing enzyme-labelled rabbit serum against mouse Igs as the second antibody (Fig. 5). As expected, the mice of the toxoid/N4 group produced antibodies against both neurotoxin (H and L components) and nonTox (lane 1), whereas the mice immunised with nonTox demonstrated no bands against neurotoxin. The mice of the nonTox/N4, nonTox/N1-O3 and nonTox/N2 groups demonstrated obvious bands with HA1, HA3a, and HA3b (especially HA1 and HA3b; lanes 2, 3, 4). In contrast, the mice of the nonTox/N1 (lane 5) and nonTox/O4 (data not shown) groups demonstrated no obvious bands to any components. When the sera from the mice of the toxoid/N4 (lane 6) or nonTox/N4 (data not shown) groups were exposed to D-16 S toxin, both sera reacted with nonTox but not neurotoxin.

Neutralisation titre of intestinal fluids and sera (neutralisation test)

The intestinal fluids and sera obtained from the mice immunised with toxoid/N4 and nonTox/N4 were diluted in serial 10-fold steps, and their ability to neutralise 10 i.p MLD of C- or D-16 S toxin in mice was observed (Table 2). The 10-fold diluted intestinal fluids and sera from nonTox-immunised mice had no neutralising activity, whereas those from toxoid-immunised mice neutralised type C toxin; the titre of sera and intestinal fluids was 10^2 and 10, respectively. These preparations did not cross-neutralise type D toxin.

Discussion

To examine the hypothesis that the nonTox components, especially HA1 and HA3b, are needed for effective absorption of the 16 S toxin from the small intestine and because the nonTox components, including HA, are almost identical between type C and D, this study investigated whether mucosal immunity against type C nonTox may prevent the absorption of both C- and D-16 S toxin from the small intestine.

Type C nonTox was purified from a mutant strain (C)-N71 that is non-toxigenic but HA-positive. Because this strain has a stop codon in the gene for the L chain of neurotoxin, it was speculated that the N-terminal region of the L chain would be obtained as well as nonTox. However, the N-terminal region of the L chain was not obtained, only whole nonTox (non-toxic non-HA and HA) and C3 enzyme (and free HA-1), which indicated that the gene of the N-terminal region before the stop codon of the L chain is not transcribed as speculated by Hauser *et al.* [19]. On SDS-PAGE, non-toxic non-HA of purified nonTox showed at least two bands with slightly different Mr, but that had the same N-terminal amino acid sequences. HA3a showed at

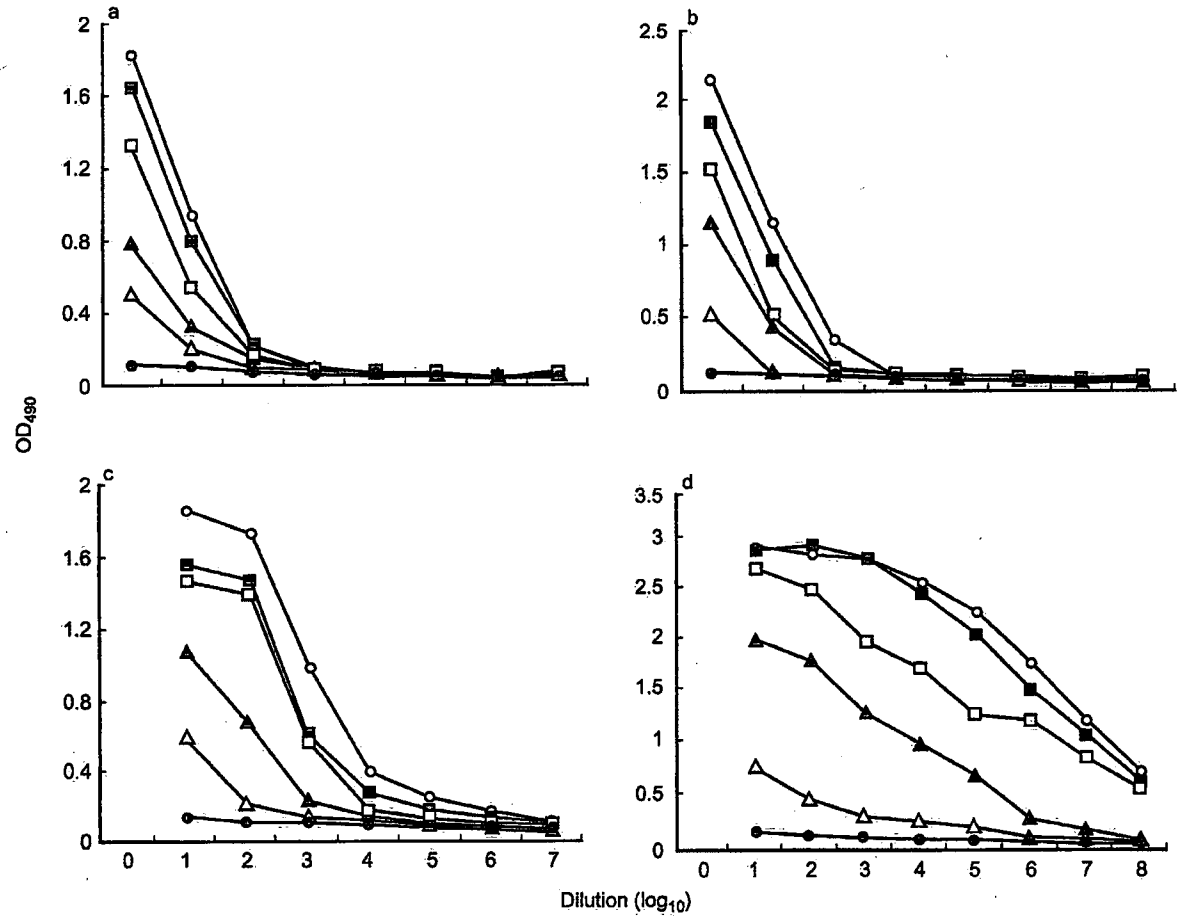

Fig. 3. ELISA of serial 10-fold dilutions of intestinal fluids and sera against (C)-N71 nonTox. IgA and total Ig titres were determined. (**a**) Intestinal fluid-IgA; (**b**) intestinal fluid-Igs; (**c**) serum-IgA; (**d**) serum-Igs. ○, toxoid/N4; ■, nonTox/N4; □, nonTox/N1-O3; ▲, nonTox/N2; △, nonTox/N1; ●, nonTox/O4.

least three different N-terminal amino acid sequences. These indicate that non-toxic non-HA and HA3b are processed after translation at their C-terminal and N-terminal regions, respectively. The latter is the same as has been described previously in types A, C and D, but the former differs from the previous findings in non-toxic non-HAs of 12 S toxins, that indicated the cleavage at their N-terminal regions [1–5]. The cleavage of C-terminal region of non-toxic non-HA of (C)-N71 might occur because this non-toxic non-HA is not conjugated with neurotoxin.

The purified nonTox and C-16 S toxoid were mixed with the mutant form of LT, and inoculated either nasally or orally into mice to establish local (mucosal) immunity. Almost all of the mice immunised with nonTox/N4, nonTox/N1-O3 and nonTox/N2 were resistant to a challenge of 2 oral MLD of either C or D. The sera and intestinal fluids of these mice showed high IgA and total Ig titres against nonTox and both C- and D-16 S toxins, but not against neurotoxin. However, the mice of the nonTox/N1 and nonTox/O4 groups were killed by the same challenge, and antibody production in both intestinal fluids and sera was poor. This indicates that the mucosal antibodies against the nonTox prevented the absorption of both C- and D-16 S toxins from the small intestine. It seems that oral administration alone is not good for inducing sufficient antibody production in this system.

When the mice immunised with nonTox and showing high antibody production (nonTox/N4, nonTox/N1-O3 and nonTox/N2 groups) were challenged with 20 oral MLD, no mice, regardless of immunisation protocol, survived although the time to death was much longer than in non-immunised control mice. This may be explained by the following three reasons: (1) some damage to mouse tissue was caused accidentally by a metal probe in inoculation of the toxin and therefore, a small amount of toxin, but enough to kill the mice, was absorbed; (2) some of the toxin was absorbed from the small intestine before they had reacted with antibodies; and (3) some of the 16 S toxin dissociates into neurotoxin and nonTox, and the neurotoxin is absorbed from the small intestine. Maksymowych *et al.* [12] reported that nonTox is not needed for toxin absorption and that neurotoxin alone can be absorbed from the stomach and the small intestine. However, in our

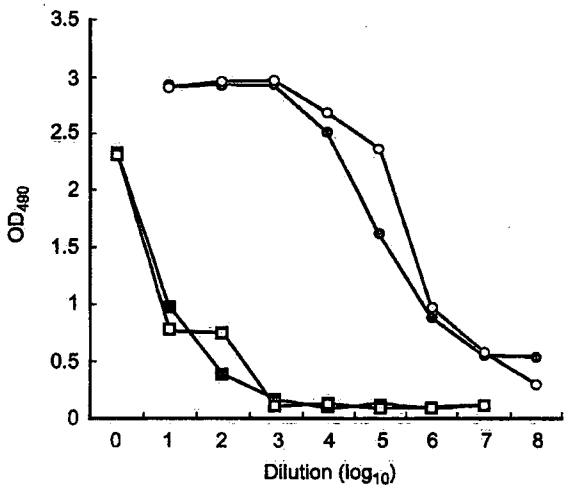

Fig. 4. ELISA of serial dilutions of intestinal fluids and sera from toxoid-immunised mice (toxoid/N4) with C- or D-16 S toxin. Rabbit serum against mouse Igs was used as the second antibody. □, Intestinal fluids against C-16 S toxin; ■, intestinal fluids against D-16 S toxin; ○, sera against C-16 S toxin; ●, sera against D-16 S toxin.

previous experiment with guinea-pigs, all three forms of type C toxin (neurotoxin, 12 S and 16 S) showed no obvious binding to the epithelial cells of the stomach. Only the 16 S toxin bound to the epithelial cells of the small intestine. When the same amounts of C-16 S, -12 S and -neurotoxin were inoculated directly into the small intestine without ligation, the guinea-pigs inoculated with 16 S died within 8 h, but those inoculated with 12 S and neurotoxin died after 12 h. Therefore, we speculated that 12 S and neurotoxin might be absorbed by a mechanism different from 16 S; one possibility is that 12 S and neurotoxin might be absorbed from M cells. Experiments are being done to clarify these points, including the conclusions proposed by Maksymowych *et al.* [12].

Only the mice immunised with toxoid (toxoid/N4) survived challenge with 20 oral MLD of type C toxin, but did not survive challenge with same dose of D-16 S toxin. This finding is reinforced by the fact that the sera and intestinal fluids of these mice had activity to neutralise type C, but not type D, toxin in the neutralisation test. They produced antibodies against type C neurotoxin in addition to nonTox.

As mentioned above, the mice immunised with nonTox survived the challenge with 2 oral MLD of both C- and D-16 S toxins that correspond to 2.4×10^4 and 8×10^3 i.p MLD, respectively. It seems that 1 oral MLD needs a relatively large amount of toxin even though 16 S toxin that is resistant to gastric and intestinal juices is employed. To be accurate, the 1 oral MLD used in these experiments is slightly higher than the true 1 oral MLD, because it was determined as the higher dilution of the toxin that killed a mouse within 1 day. However, the results obtained may not lead to the conclusion that inoculation of only the nonTox mixed with the mutant form of LT is effective against actual C and D botulism. In preliminary experiments (data not shown), mice immunised with a mixture of a large amount of nonTox and a small amount of C toxoid produced high

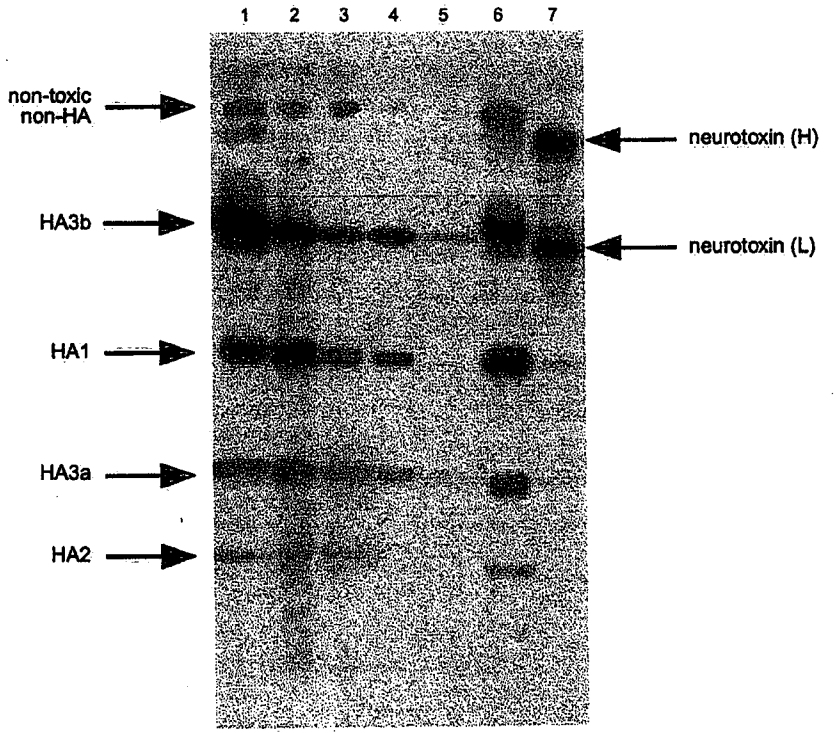

Fig. 5. Western blots of 1 in 1000 diluted sera from immunised mice with C-16 S (lanes 1–5) or to D-16 S toxin (lane 6). As a control, the reaction of anti-neurotoxin serum to C-16 S toxin is shown (lane 7). Lane 1, toxoid/N4; 2, nonTox/N4; 3, nonTox/N1-O3; 4, nonTox/N2; 5, nonTox/N1; 6, toxoid/N4.

Table 2. Toxin neutralisation test with sera and intestinal fluids from the immunised mice of (a) toxoid/N4 and (b) nonTox/N4 groups

Toxin challenge (10 i.p MLD)	Mouse lethality with serum diluted 1 in			Mouse lethality with intestinal fluid diluted 1 in	
	10	100	1000	10	100
(a)					
C	ss	ss	**dd**	ss	**dd**
D	dd	dd	ND	dd	dd
(b)					
C	dd	ND	ND	dd	ND
D	dd	ND	ND	dd	ND

Abbreviations: see footnote to Table 1.

levels of antibodies against both nonTox and neurotoxin and, therefore, were protected against challenge with 20 oral MLD of C-16 S toxin. This suggests that a mixture of a large amount of nonTox and a small amount of C and D toxoids (or H chains of their neurotoxins) can be used as a vaccine for both C and D food-borne botulism in animals and is as effective as, and safer than, toxoid. We are now planning to perform detailed vaccine experiments in birds or cattle that are frequently affected by types C and D botulism.

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References

1. Ohyama T, Watanabe T, Fujinaga Y *et al.* Characterization of nontoxic-nonhemagglutinin component of the two types of progenitor toxin (M and L) produced by *Clostridium botulinum* Type D CB-16. *Microbiol Immunol* 1995; **39**: 457–465.
2. Fujinaga Y, Inoue K, Shimazaki S *et al.* Molecular construction of *Clostridium botulinum* type C progenitor toxin and its gene organization. *Biochem Biophys Res Commun* 1994; **205**: 1291–1298.
3. Fujita R, Fujinaga Y, Inoue K, Nakajima H, Kumon H, Oguma K. Molecular characterization of two forms of nontoxic-nonhemagglutinin components of *Clostridium botulinum* type A progenitor toxins. *FEBS Lett* 1995; **376**: 41–44.
4. Inoue K, Fujinaga Y, Watanabe T *et al.* Molecular composition of *Clostridium botulinum* type A progenitor toxins. *Infect Immun* 1996; **64**: 1589–1594.
5. Inoue K, Fujinaga Y, Honke K *et al.* Characterization of haemagglutinin activity of *Clostridium botulinum* type C and D 16 S toxins, and one subcomponent of haemagglutinin (HA 1). *Microbiology* 1999; **145**: 2533–2542.
6. Oguma K, Syuto B, Iida H, Kubo S. Antigenic similarity of toxins produced by *Clostridium botulinum* type C and D strains. *Infect Immun* 1980; **30**: 656–660.
7. Oguma K, Fujinaga Y, Inoue K. *Clostridium botulinum* toxin. *J Toxicol Toxin Rev* 1997; **16**: 253–266.
8. Minton NP. Molecular genetics of clostridial neurotoxins. *Curr Top Microbiol Immunol* 1995; **195**: 161–194.
9. Ohishi I, Sugii S, Sakaguchi G. Oral toxicities of *Clostridium botulinum* toxins in response to molecular size. *Infect Immun* 1977; **16**: 107–109.
10. Ohishi I, Sakaguchi G. Oral toxicities of *Clostridium botulinum* type C and D toxins of different molecular sizes. *Infect Immun* 1980; **28**: 303–309.
11. Sugii S, Ohishi I, Sakaguchi G. Intestinal absorption of botulinum toxins of different molecular sizes in rats. *Infect Immun* 1977; **17**: 491–496.
12. Maksymowych AB, Reinhard M, Malizio CJ, Goodnough MC, Johnson EA, Simpson LL. Pure botulinum neurotoxin is absorbed from the stomach and small intestine and produces peripheral neuromuscular blockade. *Infect Immun* 1999; **67**: 4708–4712.
13. Fujinaga Y, Inoue K, Watanabe S *et al.* The haemagglutinin of *Clostridium botulinum* type C progenitor toxin plays an essential role in binding of toxin to the epithelial cells of guinea pig small intestine leading to the efficient absorption of the toxin. *Microbiology* 1997; **143**: 3841–3847.
14. Fujinaga Y, Inoue K, Nomura T *et al.* Identification and characterization of functional subunits of *Clostridium botulinum* type A progenitor toxin involved in binding to intestinal microvilli and erythrocytes. *FEBS Lett* 2000; **467**: 179–183.
15. Inoue K, Fujinaga Y, Honke K *et al.* *Clostridium botulinum* type A haemagglutinin positive progenitor toxin (HA^+-PTX) binds to oligosaccharides containing Galβ 1-4GlcNAc through one subcomponent of haemagglutinin (HA1). *Microbiology* 2001; **147**: 811–819.
16. Mahmut N, Inoue K, Fujinaga Y *et al.* Characterization of monoclonal antibodies against haemagglutinin associated with *Clostridium botulinum* type C neurotoxin. *J Med Microbiol* 2002; **51**: 286–294.
17. Oguma K, Iida H, Inoue K. Observations on nonconverting phage, c-n71, obtained from a nontoxigenic strain of *Clostridium botulinum* type C. *Jpn J Microbiol* 1975; **19**: 167–172.
18. Oguma K, Iida H, Shiozaki M. Phage conversion to hemagglutinin production in *Clostridium botulinum* types C and D. *Infect Immun* 1976; **14**: 597–602.
19. Hauser D, Gibert M, Eklund MW, Boquet P, Popoff MR. Comparative analysis of C3 and botulinum neurotoxin genes and their environment in *Clostridium botulinum* types C and D. *J Bacteriol* 1993; **175**: 7260–7268.
20. Tsuji T, Yokochi T, Kamiya H, Kawamoto Y, Miyama A, Asano Y. Relationship between a low toxicity of the mutant A subunit of enterotoxigenic *Escherichia coli* enterotoxin and its strong adjuvant action. *Immunology* 1997; **90**: 176–182.

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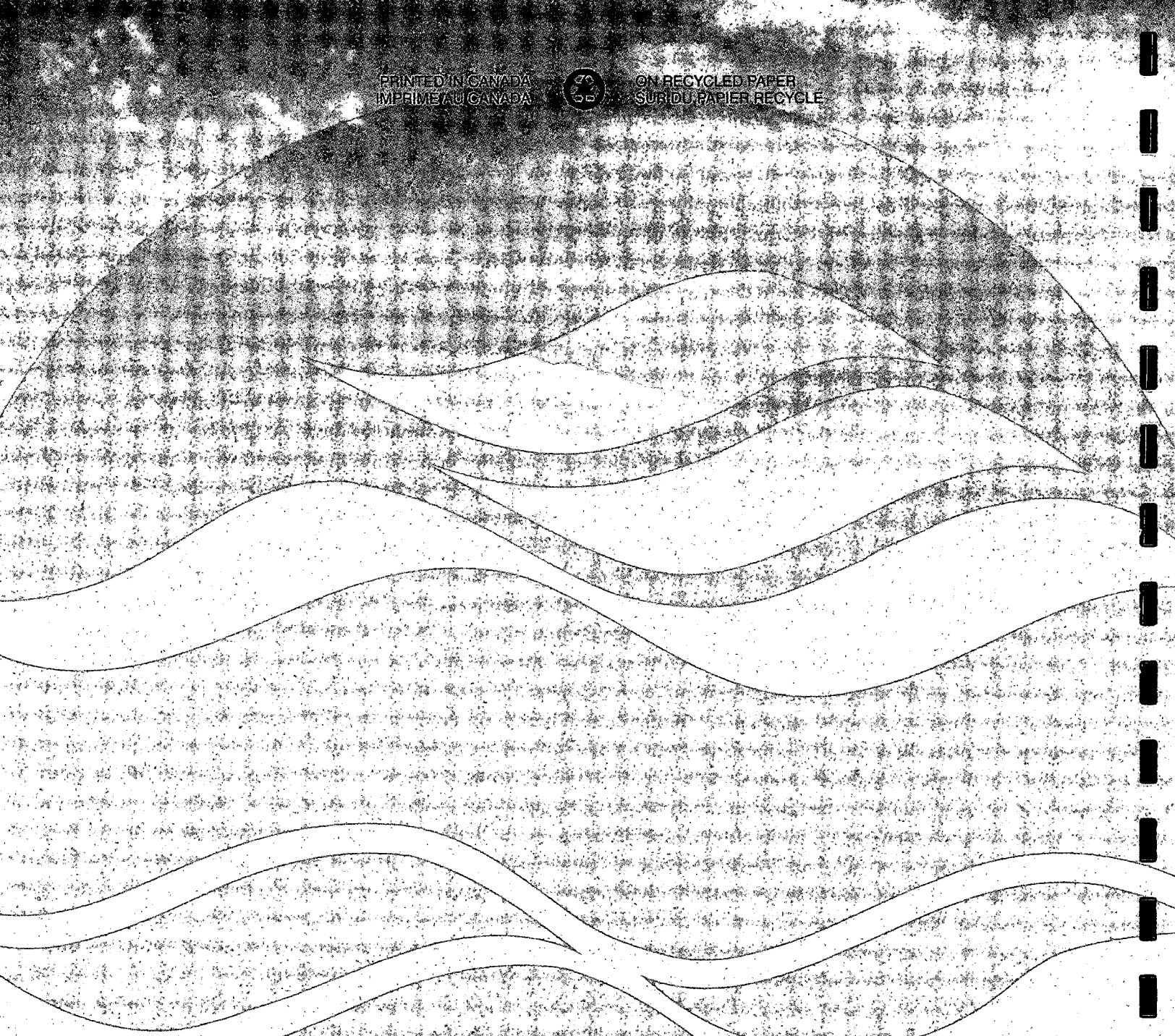

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