Studies on the *Vibrio cholerae* mucinase complex. II. Specific neuraminidase activity measured histochemically in a goblet cell assay

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Summary. The activity of neuraminidase prepared from the mucinase complex of *Vibrio cholerae* was measured by a new, semi-quantitative goblet-cell assay. The counts of normal, alcianophilic, sialomucin-containing goblet cells (purple-stained) and neutral mucosubstance-containing goblet cells (magenta-stained) in serial sections of ileum were compared before and after neuraminidase treatment. The procedure provides a more natural assessment of the action of *V. cholerae* neuraminidase on the viscous intestinal mucus.

Introduction

Sialic acid moieties (N-acylated neuraminic acid) liberated by neuraminidase from glycoproteins (mucin) or mucopolysaccharides were detected by the colourimetric thiobarbituric acid assay of Warren (1959). This test assumed that all neuraminidases would have the same activity against one substrate, bovine submaxillary mucin. Developments of this assay have centred on the use of less viscous substrates, but these have fewer carbohydrate groups per molecule, as pointed out in a previous communication (Stewart-Tull *et al.*, 1986). The more sensitive assays, such as those of Popenoe and Drew (1957), Hughes and Jeanloz (1964) or Fraser and Smith (1975), gave more accurate determination of neuraminidase activity of different micro-organisms. The substrate, N-acetyl neuraminlactose (NAN-lactose) is sensitive although expensive (Cassidy *et al.*, 1965). However, the National Institute for Allergy and Infectious Diseases (NIAID), Maryland have recommended the use of the artificial substrate 2-(3' methoxyphenol-N-acetyl α neuraminic acid) for the assay of *Vibrio cholerae* neuraminidase (see Palese *et al.*, 1973). This assay measures the liberation of blue methoxyphenol from the substrate by neuraminidase isolated from a variety of viral, bacterial and mammalian sources.

Spicer and Warren (1960) used an alcian blue/PAS staining procedure to designate neuraminidase activity as positive or negative; histochemically

there was a loss of alcianophilia in goblet cells. A similar effect was observed by Quintarelli *et al.* (1961) with bovine submaxillary mucin and rat sublingual gland mucin and by Wheeldon *et al.* (1976) with dog bronchus after treatment with influenza virus neuraminidase.

During studies of the mucinase complex of *V. cholerae*, the relation between the quantitative, *in vitro*, colourimetric assays of neuraminidase activity and the histochemical reaction in tissue sections was examined. This paper describes a new goblet-cell assay based on the numerical difference between the alcianophilic, normal goblet cells and magenta-staining cells containing neutral mucosubstances that remain after treatment with neuraminidase.

Materials and methods

Vibrio cholerae NCTC 10732, the classical biovar, Inaba serovar, was obtained from the National Collection of Type Cultures, Colindale, London.

Production and purification of *V. cholerae* mucinase complex and estimation of neuraminidase activity. These procedures were described in detail by Stewart-Tull *et al.* (1986). The methanol precipitate of the crude culture filtrate of *V. cholerae* was purified by Sephadex G100 filtration. The fractions, eluted with 0.05 M sodium acetate buffer, pH 5.5, that had neuraminidase activity were pooled and concentrated and this material is referred to as G100/N.

Cholera enterotoxin. Crude *V. cholerae* culture filtrate containing enterotoxin was kindly provided by the Research Resources Branch, NIAID, Bethesda, MD, USA.

Commercial neuraminidase preparations. *Clostridium perfringens* neuraminidase EC 3.2.1.18 was obtained from Sigma Chemical Co., Poole, Dorset; this has a potency

of 680 international milli-units (ImU)/ml and may contain aldolase and proteinase. An equivalent product from Worthington (Millipore UK Ltd, London) had a potency of 680 ImU/ml and contained <0.1% proteolytic activity. *V. cholerae* neuraminidase preparations were obtained from (1) Koch-Light Laboratories, Suffolk (1 IU/ml; no detectable aldolase, phospholipase or proteinase) and (2) Calbiochem-Behring, Cambridge Bioscience, Cambridge (1 IU/ml; contains aldolase <10 mU/ml, phospholipase <20 mU/ml, and proteinases <0.1 mU/ml).

Histological procedures. The ileum was removed from a healthy guinea pig, rat or rabbit. Ileal segments were wrapped in aluminium foil and quickly frozen in liquid nitrogen. The frozen tissue segments were stored at -170°C , and subsequently removed from the foil and cut transversely into small lengths. Each segment was placed transversely in an aluminium foil boat (2×3 cm) filled with Ames OCT freezing compound (Raymond Lamb Ltd, London). The foil boat was kept in an isopentane slurry, cooled with liquid nitrogen, until the tissue turned completely milky-white. The tissue block was removed and fixed to a cryostat chuck-face. Serial sections, 8 μm thick, cut in a cryostat (SLEE Medical Ltd, London) at -25°C , were laid on glass microscope slides (precleaned with ethanol 90% v/v and coated with ovalbumin 0.1% w/v) and placed at 4°C to thaw slowly.

Preservation of mucosubstances by paraformaldehyde vapour. The tissue fixation procedure was basically as described by Pearse and Tock (1965). A desiccator was completely dried in an 80°C oven; a crucible, containing paraformaldehyde powder (BDH) 5 g, was placed inside the desiccator at room temperature. The lid was replaced and the vessel was heated at 80°C . After 1 h, the crucible was removed quickly and replaced with a tray of microscope slides holding sections of ileal tissue. The closed desiccator was kept at 60°C for 1 h and the slides were then removed for staining.

Alcian blue-periodic acid Schiff histochemical staining method (AB/PAS). This was as described by Mowry and Winkler (1956). Sections were stained in alcian blue (Raymond Lamb, London; AB 10 g in 30 ml of concentrated acetic acid made up to 100 ml with distilled water) at pH 2.5 for 30 min. After washing in running water, the sections were treated with aqueous HIO_4 1% v/v for 10 min and washed again. The sections were counter-stained with Schiff's reagent [pararosaniline chloride (Kodak Ltd) 1% w/v decolourised with sulphur dioxide gas] for 10 min, rinsed three times for 2 min in sodium metabisulphite 9.5% w/v, rinsed in water, dehydrated, cleared and mounted in DPX.

The effect of neuraminidase and other chemical treatments on goblet-cell histochemistry

Two frozen paraformaldehyde-fixed transverse sections of rat ileum were treated for 14 h at 37°C in a moistened chamber with 0.2 ml of one of the following: (i) distilled water; (ii) 0.05 M sodium acetate (NaAc) buffer, pH 5.0 or 5.5; (iii) *C. perfringens* neuraminidase (Worthington); (iv) *C. perfringens* neuraminidase

(Sigma); (v) *V. cholerae* partially purified neuraminidase G100/ N_2 (55 ImU/ml); (vi) *V. cholerae* neuraminidase (Calbiochem); (vii) concentrated trypsin inhibitor (Sigma; 1.0 mg/10 ml of 0.05 M NaAc buffer, pH 5.5); (viii) concentrated trypsin inhibitor (2.0 ml) + neuraminidase G100/ N_2 (0.1 ml) + 0.05 M NaAc buffer, pH 5.5 (0.05 ml); (ix) borate gelatin buffer, pH 7.5; (x) cholera enterotoxin (NIAID; 3.0 g/5.0 ml of borate gelatin buffer, pH 7.6); (xi) 0.05 M NaAc buffer, pH 5.5 (0.3 ml) + 0.01 M phosphate buffer, pH 7.5 (0.05 ml); (xii) 0.05 M NaAc buffer, pH 5.5 (0.05 ml) + 0.01 M phosphate buffer, pH 7.5 (0.3 ml). Two frozen sections were left untreated to serve as additional controls.

Goblet-cell assay. The sections were examined by light microscopy and goblet cells were counted in 20 random fields. AB-positive cells were stained blue (periodate-unreactive) or purple (periodate-reactive acid mucosubstances), whereas PAS-positive cells were stained magenta (neutral periodate-reactive mucosubstances). The data were analysed statistically by Student's *t* test (two-tailed); the goblet-cell counts in the untreated control or buffer-treated sections were compared with those in sections treated with neuraminidase preparations.

Results

The effect of V. cholerae neuraminidase on goblet-cell histochemistry

The untreated and neuraminidase-treated rat ileal-tissue sections were examined by light microscopy. In areas where the villi were in transverse section, distinct whorls of goblet cells were seen. In the control rat ileal section, the cells in these whorls were stained predominantly blue/purple and there were fewer magenta-stained cells (plate *a* and *c*). However, when serial sections were treated with different amounts of *C. perfringens* or *V. cholerae* neuraminidase, there was a distinct change in the histochemical reaction and the goblet cells were stained predominantly magenta (plate *b* and *d*). A similar effect was noticed with longitudinal sections of villi. Consequently, counts were made of the goblet cells that were stained either blue/purple or magenta by the alcian blue/PAS method. The number of cells stained by alcian blue was 553/cm² field area in the untreated section. However, after treatment with neuraminidase for 24 h at 37°C , there were only 282/cm². Similarly, the numbers of magenta PAS-stained cells were 119/cm² in the untreated and 193/cm² in the neuraminidase-treated sections.

Statistical analyses revealed significant differences in the alcian blue counts ($p < 0.0002$) and PAS counts ($p < 0.001$) for the untreated and neuraminidase-treated preparations. There was no significant difference in the respective counts for

Plate. *Rat ileum*, longitudinal sections; *a, c* are untreated controls; *b, d* were treated with Worthington *C. perfringens* neuraminidase 136 ImU for 24 h at 37°C. Sections were stained with alcian blue/PAS at pH 2.5. Note the darker alcianophilic goblet cells (*a, c*, purple-stained) in the untreated sections and the lighter staining neutral mucosubstances in goblet cells treated with neuraminidase (*b, d*, magenta-stained). (*a, b* × 465, *c, d* × 656)
Rabbit ileum, transverse sections; *e, g* are untreated controls; *f* was treated with Koch-light *V. cholerae* neuraminidase 500 ImU, and *h* was treated with *V. cholerae* G100/N₂ for 24 h at 37°C. Some goblet cells in *h* were evaginated after neuraminidase treatment. (*e, f* × 130; *g, h* × 656)

untreated sections and those treated with 0.05 M NaAc buffer, pH 5.5, used as the diluent for the neuraminidase.

Histochemical reactions were similar with rat, guinea-pig or rabbit ileal sections treated with *V. cholerae* neuraminidase preparations (plate, e-h). Generally, rat ileum produced a better alcian blue/PAS histochemical reaction and was economical to obtain, so further experiments were done with this material.

The effect of different neuraminidase preparations on the goblet-cell assay

The neuraminidase activities of four commercial enzyme preparations from *C. perfringens* or *V. cholerae* were compared with that of the *V. cholerae* G100/N mucinase preparation. The most active commercial neuraminidase was derived from *C. perfringens*; 136 ImU of the Sigma preparation reduced the alcian blue reaction for NANA (sialic acid, N-acetylneuraminic acid) from 555/cm² to 68/cm² (figure). A similar quantity of the Worthington preparation was less active and reduced the count to 283/cm² (figure).

With the *V. cholerae* preparations, the number of cells stained by alcian blue was 111/cm² for 100 ImU of the Calbiochem enzyme and 210/cm² for 11 ImU of the partially-purified *V. cholerae* G100/N₂ mucinase complex (figure).

Statistical analyses showed a significant difference between the alcian blue counts for the untreated sections and in those exposed to the neuraminidase preparations ($p < 0.0002$).

Discussion

Stewart-Tull *et al.* (1986) showed that the thiobarbituric acid (TBA) assay was less sensitive than the methoxyphenol-neuraminase (MPN) assay as a monitor of specific neuraminidase activity in the *V. cholerae* mucinase complex. Montgomery (1970) discussed the isolation, fractionation and purity of glycoproteins and pointed out that it is difficult to prove that preparations are free from extraneous material. This is particularly true for the highly-charged mucins that produce viscous solutions. In such viscous solutions the neuraminidase may be prevented from reaching sites of specific action with a consequent lower level of NANA released. Furthermore, the number of carbohydrate groups per molecule of glycoprotein may influence the result of the in-vitro colourimetric assay.

Similarly, Aminoff (1961) noted that the reaction of neuraminic acid in the TBA assay was identical to that obtained with 2-oxo-3-deoxy sugar acids. Several other carbohydrate residues will give positive reactions in the TBA assay, including 2-deoxyribose, 3-deoxy- and 3,6 dideoxyhexoses,

Figure. Counts of goblet cells in 20 random fields of rat ileum sections treated with various neuraminidase preparations. Section 1, control untreated; 2, treated with Worthington *C. perfringens* enzyme 136 ImU; 3, treated with Sigma *C. perfringens* enzyme 136 ImU; 4, treated with Calbiochem *V. cholerae* enzyme 100 ImU; and 5, treated with *V. cholerae* G100/N₂ enzyme 11 ImU. The solid columns represent the number of normal alcianophilic goblet cells (purple-stained) and the open columns the number of goblet cells containing neutral mucosubstances after neuraminidase treatment (magenta-staining).

together with the residues released by neuraminidase and endoglycosidase in the mucinase complex.

For these reasons, the use of the substrates N-acetylneuraminlactose (Cassidy *et al.*, 1965) or 2-(3-methoxyphenyl-N-acetyl α neuraminic acid) (Palese *et al.*, 1973) has been recommended for the in-vitro assay of neuraminidases. However, this type of assay provides sparse information about the role of the *V. cholerae* neuraminidase in the gut. The alcian blue/PAS histochemical staining procedure has been used to show that neuraminidase produced *in situ* will destroy the alcianophilia of the goblet cells. The present study has extended the work on the qualitative nature of the histochemical effect and it has been possible to quantitate the activity of the neuraminidase in the environment of a semi-natural experiment.

When unfixed frozen sections of rat ileum were used in studies to detect the presence of the neuraminidase in the *V. cholerae* mucinase complex, it was found that the aqueous solutions of the histochemical stains removed the neuraminidase substrate mucin. A similar problem was noted by Pearse and Tock (1965) and Tock and Pearse (1965) with liquid, chemical fixation procedures. The formaldehyde vapour method of Pearse and Tock (1965), as described by Culling (1974), considerably reduced the non-enzymic loss of intestinal mucin and preserved the goblet-cell mucin. This enabled the effect of *V. cholerae* neuraminidase to be assayed by the alcian blue/PAS reaction.

It was noticed also that the most distinct histochemical reaction occurred in sections of rat ileum after neuraminidase treatment. Forstner *et al.* (1973) isolated and chemically examined the

goblet-cell mucin from rat intestines; after neuraminidase treatment (Sigma type VI, *C. perfringens*), 86.7% of the total sialic-acid residues were released from the rat goblet-cell mucin. Therefore, it seems likely that this native glycoprotein has readily accessible sialic-acid terminal carbohydrate residues that are susceptible to *V. cholerae* neuraminidase. In our study the neuraminidases of both *C. perfringens* and *V. cholerae* were active against rat goblet-cell mucin. Although Ramphal and Pyle (1983) found a similar effect with bovine sub-maxillary mucin, only the *V. cholerae* enzyme released NANA from mouse tracheal tissue. Whether these different findings can be attributed to differences in the steric configuration of the target sialic acid in the glycoprotein or to slight differences in enzyme specificity remains to be determined.

Forstner *et al.* (1981) found that cholera enterotoxin stimulated the secretion of sialomucin from the intestinal surface. In our study, treatment of rat ileal sections, fixed in paraformaldehyde vapour, with purified cholera enterotoxin, as expected, did not significantly alter the number of alcianophilic goblet cells. Nevertheless, the assay may be useful in monitoring the hypersecretion of sialomucin after intestinal stimulation with enterotoxin *in vivo*.

The goblet-cell histochemical assay provides a useful complement to the more artificial colourimetric assays, although the latter are more rapid and less substrate specific. The specificity of the alcian blue reaction for neuraminic acid proved that other studies with anti-neuraminidase antisera were confused totally by the presence of serum sialidase.

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