The Separation of Neuraminidase Activity from other Pathological Activities of a Culture Filtrate of *Clostridium sordellii* CN 3903

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SUMMARY

The neuraminidase activity of a culture filtrate of *Clostridium sordellii* CN 3903 was separated on a Sephadex column from the oedema-producing, haemorrhage-producing and lecithinase activities. It is concluded that the neuraminidase of *C. sordellii* is not able by itself to produce oedema and haemorrhage.

INTRODUCTION

Neuraminidases (*N*-acetylneuraminic glycohydrolase, E.C. 3.2.1.18) have been demonstrated in many micro-organisms (see, for example, *Vibrio cholera*, Gottschalk, 1957; *Diplococcus pneumoniae*, Madoff, Eylar & Weinstein, 1960; *Corynebacterium diphtheriae*, Warren & Spearing, 1963; some group K streptococci, Hayano & Tanaka, 1967; several human oral bacteria, Thonard, Hefflin & Steinberg, 1965; some myxoviruses, Rafaelson, Schneir & Wilson, 1963; *Clostridium perfringens*, Popenoe & Drew, 1957; Cassidy, Jourdan & Roseman, 1965). Neuraminidases cleave the glycosidic linkage of neuraminic acid in several types of compounds, including glycoproteins and gangliosides. Since these compounds appear to be components of cell membranes, and the neuraminic acid probably contributes to the charge on the membrane surface, neuraminidases might be expected to produce profound changes when introduced into living material. Although the receptor-destroying activity of viral neuraminidases has been known for a long time, no pathological activity of a micro-organism has yet been established to be due to a neuraminidase. Thus Kelly, Grieff & Farmer, (1966) found no correlation between animal virulence of *D. pneumoniae* and its ability to produce neuraminidase; Burton (1963) reported that the neuraminidase of *C. perfringens* was non-toxic to mice; Collee (1965; Gadalla & Collee 1968) was able to show that the haemagglutinin and neuraminidase of *C. perfringens* and of *C. septicum* were separate proteins; Fuhrman & Fuhrman (1968) reported that a factor in culture filtrates of *Vibrio cholera* which inhibited the sodium pump of frog skin differed from the cholera neuraminidase.

Cell extracts and culture filtrates of *Clostridium sordellii* are toxic, containing factors which produce oedema and haemorrhage when injected into guinea-pig skin. We have shown (Mellanby & White, 1968) that these pathological actions are independent of a lysolecithinase which the organisms produce intracellularly in the early stages of growth. At this early stage the culture filtrates contain about 1000 mouse LD₅₀/ml. and neuraminidase activity of about the same specific activity as found in

C. perfringens culture filtrates (see Cassidy *et al.* 1965). The work reported in the present paper comprises a separation on Sephadex of some of the activities of a *C. sordellii* culture filtrate, in an attempt to assess whether the neuraminidase can be correlated with its pathological activity.

METHODS

Lecithin (33% pure from egg), sodium deoxycholate, laboratory reagent, glycylglycine, laboratory reagent, were obtained from British Drug Houses Ltd., Sephadex G-75 was obtained from Pharmacia (Uppsala, Sweden). The ganglioside was prepared from beef brain by the method of Mellanby, Pope & Ambache (1968).

Preparation of concentrated Clostridium sordellii culture filtrate. The *C. sordellii* (strain CN 3903, kindly provided by the Wellcome Research Laboratories) was grown for 4 hr at 37° in 10 l. bottles in a medium containing 30 g. Oxoid Bacteriological Peptone, 7.5 g. Na- β -glycerophosphate and 0.1 ml. of neutralized thiolacetic acid (approximately 0.3 m-mole) per litre. It was inoculated with an inoculum grown overnight in cooked-meat medium, which had itself been inoculated with 1 drop/10 ml. of a spore suspension (containing 100 mg. dry weight/ml.) stored in 50% glycerol at -40°. The organisms were removed by centrifugation at 10,000 *g* for 10 min. The 10 l. of supernatant fluid were concentrated to 200 ml. by pressure dialysis at 4° during a period of several weeks. The protein in the concentrated liquid was precipitated by adding ammonium sulphate (350 g./l.) at 4°, maintained at pH 7. The mixture was allowed to stand for 30 min. and then centrifuged at 17,000 *g* for 30 min. The precipitate was dissolved in 25 ml. cold distilled water and dialysed overnight against 10 l. distilled water at 4°. The dialysed solution was then freeze-dried; this material was stored at 4° and used for the subsequent work.

Assay of neuraminidase activity. In a total volume of 0.2 ml. 0.75 mg. ganglioside in 0.2 M-acetate buffer (pH 5.5) was incubated with the sample of enzyme for 10 min. at 37° and the free neuraminic acid at the end of the incubation estimated by the method of Warren (1959). One unit of neuraminidase activity was defined as that amount which released 1 μ mole neuraminic acid from ganglioside per minute.

Assay of lecithinase-A activity. Lecithin and sodium deoxycholate (10 mg./ml. each) were suspended in 0.1 M-glycylglycine buffer (pH 7.2) and the mixture treated ultrasonically (MSE 100 W ultrasonic disintegrator) for 30 sec. to produce a homogeneous suspension. This mixture (0.3 ml.) was incubated with the enzyme sample in a total volume of 1 ml. at 37° for 6 hr. The reaction was stopped by placing the tubes in ice and adding 5 ml. of extraction mixture (isopropanol + *n*-heptane + 2 N-H₂SO₄; 80 + 20 + 1 by vol.). Free fatty acids were then titrated by using the method described by Kelley (1965). One unit of lecithinase activity was defined as the amount of enzyme that released 5×10^{-3} μ mole fatty acid/hr (Mellanby & White, 1968).

Pathological activity of culture filtrate fractions in rabbit skin. Serial dilutions of samples (0.1 ml. in 0.1 M-phosphate buffer, pH 7, containing 0.2% (w/v) gelatin) to be tested were injected intradermally into the shaved back of albino rabbits (3 kg.) anaesthetized with Nembutal. Five and a half hr later the rabbits were injected in the ear vein with 1.5 ml. of Pontamine Sky Blue solution (0.5% in 0.45% NaCl). Half an hour after this the diameters of the blue oedematous areas were measured. One unit of oedema-producing activity was defined as that required to produce 10 mm. diameter oedema in rabbit skin after 6 hr (see Arseculeratne, 1965).

RESULTS

Separation on Sephadex of activities in concentrated culture filtrate

The material prepared from concentrated culture filtrate (60 mg.) dissolved in 2 ml. 0.001 M-phosphate buffer (pH 6.0) was applied to a Sephadex column (2.6 × 28 cm.) and eluted in 2.5 ml. fractions with the same buffer. The fractions were assayed for protein, lecithinase, neuraminidase, lethality in mice and ability to produce oedema and haemorrhage in guinea-pig skin. The results obtained are shown in Fig. 1. It can

Fig. 1. Separation of activities of *Clostridium sordellii* CN 3903 culture filtrate on Sephadex. x—x, Upper curve: mg. needed to give 1 unit oedema-producing activity; lowest curve: protein mg./ml.

be seen that protein was eluted in a steep initial peak followed by a gradual slope. The initial protein peak contained the lecithinase activity and the pathological activity, as manifested in the guinea-pig skin and lethal-toxicity to mice. The material which emerged after the initial protein peak was non-toxic to mice. The early part of the second increase in protein content contained a fraction with the neuraminidase

activity. The tube which contained the greatest amount of neuraminidase activity contained no detectable pathologically active material. It could therefore be concluded that the neuraminidase of *Clostridium sordellii* is not able by itself to produce oedema and haemorrhage. The lecithinase activity paralleled the pathological activity and therefore possible may be responsible for it. Table 1 shows that the procedure resulted in a modest purification of the neuraminidase (about 2½-fold) and of the lecithinase (about 3-fold) activities. The separated neuraminidase had an optimum activity at pH 5.0, while the lecithinase had an optimum activity at pH 7.2.

Table 1. *Recovery and specific activity of neuraminidase, lecithinase and oedema-producing activity on passing a preparation of Clostridium sordellii culture filtrate through Sephadex*

Fraction	Total protein (mg.)	Neuraminidase* activity		Lecithinase† activity		Oedema-producing activity‡	
		Total units	Units/mg. protein	Total units	Units/mg. protein	Total units	Units/mg. protein
Original	60	2.5	0.04	1440	24	36	0.6
Neuraminidase peak (tubes 17-23 inc.)	11.3	1.3	0.11	0	—	0	—
Lecithinase and toxic peak (tubes 11-15 inc.)	7.4	0	—	510	69	24	3.4

* One unit of neuraminidase activity released 1 μ mole of neuraminic acid per minute (see Methods).

† One unit of lecithinase activity released 5×10^{-8} μ mole fatty acid per hr (see Methods).

‡ One unit of oedema-producing activity gave a 10 mm. diameter oedematous lesion in rabbit skin after 6 hr (see Methods).

In view of the suggestion of Gadalla & Collee (1968) that neuraminidase might increase the pathological activity of other necrotic toxins without itself being pathologically active, the oedema-producing activity of the most active fractions obtained above was compared, with and without addition of the neuraminidase. However, up to 0.01 units of neuraminidase activity had no effect on the oedema-producing or haemorrhagic activity.

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