The Relation between the Tetanus Toxin-Fixing and Influenza Virus-Inhibiting Properties of Ganglioside

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SUMMARY

The capacity of a number of gangliosides to fix tetanus toxin and to inhibit haemagglutination by influenza virus was investigated. Toxin fixation increased with total sialic acid content but not in strict proportion. Whether or not the sialic acid is bound by neuraminidase-labile linkage appears to be irrelevant in determining whether toxin is fixed. Viral inhibition by ganglioside also is related to the total content of sialic acid; but there exists at least one ganglioside which has very little capacity to react with virus and whose sialic acid is virtually all insensitive to neuraminidase. Tetanus toxin does not prevent neuraminidase from attacking ganglioside.

INTRODUCTION

Ganglioside from nervous tissue has been shown to act as a specific receptor for tetanus toxin (Van Heyningen, 1959) and the sialic acid residue(s) of this compound has been shown to be essential for toxin-fixation (Van Heyningen & Miller, 1961). It is well known that sialic acid-containing substances also act as influenza virus receptors. Rosenberg, Howe & Chargaff (1956) found that their preparation of mucolipid, which contained a polypeptide moiety in addition to ganglioside, inhibited influenza virus, and was susceptible to viral and cholera neuraminidase, whereas samples of ganglioside which did not contain amino acid residues were inactive. They considered that the polypeptide moiety, in addition to the sialic acid residue, was essential for viral inhibitory activity. It has been observed that amino acids are present in ganglioside preparations extracted from wet brain, but not in preparations extracted from acetone-dehydrated brain (Folch, Arsove & Meath, 1951; Svennerholm, 1956). Bogoch (1957) showed that a preparation of brain ganglioside from wet brain inhibited viral haemagglutination. Kuhn (1958) separated two fractions of brain ganglioside from dehydrated brain on a cellulose column eluted with methanol + pyridine + water and found that the more rapidly moving fraction (G 2), containing 21 % sialic acid, did not inhibit viral haemagglutination and was not susceptible to *Vibrio cholerae* neuraminidase; the more slowly moving fraction (G 0.5) containing 31 % sialic acid, inhibited viral haemagglutination and was susceptible to neuraminidase. Van Heyningen & Miller (1961) separated brain ganglioside (prepared from dehydrated brain and containing no amino acid

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residues) into 'fast' and 'slow' fractions from silicic acid columns eluted with methanol + chloroform. The 'fast' preparation contained $0.75 \mu\text{mole}$ sialic acid/mg. and the 'slow' $0.98 \mu\text{mole}$ /mg. The tetanus toxin-fixing capacities of these fractions were approximately proportional to their sialic acid contents. They also showed that the mucolipid preparation of Rosenberg *et al.* (1956) was 'slow' and contained $0.89 \mu\text{mole}$ sialic acid/mg., whereas an amino acid-free preparation supplied by Professor E. Klenk was 'fast' and contained $0.66 \mu\text{mole}$ sialic acid/mg. The tetanus toxin-fixing capacities of these preparations were also roughly proportional to their sialic acid contents. We have now investigated the influenza virus-inhibiting properties of these various ganglioside preparations in relation to their toxin-fixing capacities.

Fig. 1. Paper chromatograms of various ganglioside preparations. Components 1 and 2 metachromatic, 3 non-metachromatic.

METHODS

Ganglioside preparations. The preparation (or source) and properties of the 'partially purified', 'fast', 'slow', 'Chargaff' and 'Klenk' gangliosides are described in the preceding paper (Van Heyningen & Miller, 1961). The G 2.0 and G 0.5 preparations were kindly supplied by Professor Richard Kuhn. Their paper chromatographic behaviour in comparison with 'fast' and 'slow' ganglioside preparations is shown in Fig. 1.

Paper chromatography. As described by Van Heyningen & Miller (1961), except that for Fig. 3 the ratio of di-*isobutylketone*, acetic acid, water was 40:25:5, rather than 40:30:7.

Assays. Total sialic acid was estimated by the resorcinol method of Svennerholm (1957); free sialic acid by the thiobarbituric acid assay of Warren (1959); tetanus-toxin fixing capacity (RU/mg.) by the method of Van Heyningen & Miller (1961); influenza virus-inhibiting capacity essentially by the method of Howe (1951). The PR8 strain of influenza virus was harvested from eggs, the combined allantoic fluid centrifuged and the sedimented virus resuspended in 0.1 vol. 0.15 M-NaCl. Virus prepared in this way (haemagglutinin titre 64,000) was kindly supplied by Dr A. Isaacs. Indicator virus was prepared by heating this preparation for 30 min. at 55–56°. The titrations were carried out in MRC pattern Perspex plates. Serial two-fold dilutions of ganglioside contained in 0.2 ml. 0.15 M-NaCl, buffered to pH 7.2 with 0.01 M-phosphate, were mixed with 1–2 haemagglutinin units of indicator virus and allowed to stand in the refrigerator for 30 min.; 0.1 ml. of a 2% suspension of washed chicken erythrocytes was then added, and after standing 1–2 hr. in the cold, the patterns of the sedimented erythrocytes were observed. The end-point used was the smallest amount of ganglioside giving just short of complete inhibition of haemagglutination.

Neuraminidase. Two preparations were used: (a) crude freeze-dried *Vibrio cholerae* filtrate (N. V. Philips-Roxane, Amsterdam), (b) crystalline *V. cholerae* neuraminidase kindly supplied by Dr G. L. Ada. Neuraminidase (RDE) activity was estimated by the method of Burnet & Stone (1947). The Ca + acetate + saline used in experiments with neuraminidase contained per l. distilled water: 12.38 g. sodium acetate, 5.0 g. NaCl, 1.0 g. CaCl₂.H₂O; adjusted to pH 6.2 with 2 N-acetic acid.

RESULTS

Inhibition of haemagglutination

The viral haemagglutination-inhibiting property of the various ganglioside preparations is shown in Table 1.

Action of neuraminidase on ganglioside

The thiobarbituric acid assay (Warren, 1959) is useful for the measurement of enzymic liberation of sialic acid from ganglioside since it estimates only free sialic acid. Figure 2*a* shows the time course of the liberation of sialic acid from 'slow' ganglioside by crude *Vibrio cholerae* neuraminidase (75 units/ml.). Maximal splitting was reached in about 8 hr. Figure 2*b* shows the liberation of sialic acid from 'slow' ganglioside by increasing concentrations of crystalline neuraminidase in 16 hr. Maximal splitting was obtained with 75–100 enzyme units/ml.

Comparative susceptibility of various ganglioside preparations to neuraminidase. Neuraminidase-labile sialic acid of the several ganglioside preparations was estimated by incubating 160 µg. ganglioside with 4000 units of crystalline neuraminidase in a volume of 0.4 ml., pH 6.2, at 36°. After 16 hr. free sialic acid was measured (Warren, 1959) and the values obtained corrected for small amounts of colour and turbidity given by untreated ganglioside. The results are shown in Table 1.

Table 1. *Chromatographic behaviour, neuraminidase-sensitivity, viral-inhibition and toxin-fixation of ganglioside preparations*

Ganglioside preparation	Chromatographic properties of main component(s)		Total sialic acid (μ mole/mg.)	Fraction of total sialic acid liberated by neuraminidase (%)	Amount required to inhibit 1-2 haemagglutinin units of influenza virus (μ g.)	Toxin-fixing capacity	
	Movement	Staining with cresyl violet				RU/mg.	RU/ μ mole sialic acid
Partially purified	Fast and slow	N and M*	0.73	44	8	1100	1505
Fast	Fast	N	0.75	35	8	950	1280
Slow	Slow	M	0.93	57	1	1310	1400
Kuhn G0.5	Slow	M	0.82	33	0.5	820	1000
Kuhn G2.0	Fast	N	0.58	2	20	580	1000
Chargaff mucolipid	Slow	M	0.89	47	0.5	1190	1340
Klenk ganglioside	Fast	N	0.66	21	10	540	820

*M = metachromatic, i.e. stains pink-mauve; N = non-metachromatic, i.e. stains blue-mauve.

A preparation of influenza virus, 50 times as concentrated (the greatest practicable) as the allantoic fluid from which it was derived, released 48 % of the total sialic acid of the 'slow' ganglioside in 16 hr. at 36° (compare 57 % released by *Vibrio cholerae* neuraminidase).

Fig. 2a. Course of appearance of sialic acid in a mixture of ganglioside and *Vibrio cholerae* filtrate. One ml. crude *V. cholerae* filtrate reconstituted to twice its original volume with calcium + acetate + saline (pH 6.2) was added to one ml. of the same diluent containing 0.8 ml. 'slow' ganglioside. During incubation at 36°, 0.2 ml. samples were removed at the times indicated and delivered into tubes containing 0.1 ml. sodium periodate in phosphoric acid, and free sialic acid subsequently estimated. The values are corrected for colour given by the *V. cholerae* filtrate.

Fig. 2b. Liberation of sialic acid as a function of enzyme concentration. Each point represents the free sialic acid found after incubating for 16 hr. at 36° 80 μg. 'slow' ganglioside in 0.1 ml. calcium + acetate + saline (containing 0.1 % gelatin) + 0.1 ml. crystalline *V. cholerae* neuraminidase in same diluent. The values are corrected for the small amount of colour given by the ganglioside alone.

Fixation of tetanus toxin

The tetanus toxin-fixing capacities of the various ganglioside preparations are shown in Table 1. It is evident that even when practically none of the sialic acid was neuraminidase-sensitive (as in preparation G 2·0) ganglioside still fixed toxin. The data suggest that neuraminidase-insensitive as well as neuraminidase-sensitive sialic acid is concerned in toxin-fixation. In order further to test this point a solution

Fig. 3. Paper chromatogram of 'slow' ganglioside after treatment with neuraminidase (*a*) compared with partially purified ganglioside (*b*). Components 1 and 2 metachromatic, 3 non-metachromatic.

containing 1·2 mg. 'slow' ganglioside and 6000 units neuraminidase in 3 ml. Ca + acetate + saline was incubated for 16 hr., with liberation of 48 % of the total sialic acid. The mixture was dialysed overnight against running tap water and freeze-dried. The product was found to contain 580 RU/mg., or 1140 RU/ μ mole sialic acid.

Figure 3 shows a paper chromatogram of the neuraminidase-treated preparation,

compared with unfractionated ganglioside. It can be seen that the neuraminidase had converted 'slow' ganglioside into 'fast' ganglioside.

The possibility was tested that tetanus toxin might competitively inhibit the action of neuraminidase on ganglioside, but as much as 2.6 mg. toxin/ml. did not significantly diminish the rate of liberation of sialic acid from ganglioside as compared to that of neuraminidase acting on ganglioside in the absence of toxin. The concentration of toxin was sufficient to combine with all the ganglioside present; the concentration of neuraminidase was *c.* 0.01 μ g./ml. We were unable to test the reverse concentrations of toxin and neuraminidase because of the limited supply of pure neuraminidase at our disposal.

DISCUSSION

Differences in the ability of various gangliosides to inhibit viral haemagglutination appear to reside in differences in sialic acid content rather than in the presence or absence of amino acid residues as suggested by Rosenberg *et al.* (1956). Not all of the sialic acid which is responsible for viral inhibition is necessarily neuraminidase-sensitive, for comparison of the G0.5 and 'slow' preparations shows the proportion of neuraminidase-sensitive sialic acid to be much lower in G0.5 than in 'slow', while the latter, if anything, had slightly less viral inhibitory capacity.

The gangliosides having a greater sialic content are 'slow' and metachromatic on paper chromatography, and Figs. 1 and 3 (as well as Fig. 6 in Van Heyningen & Miller, 1961) show that there are apparently two such gangliosides. There is a suggestion in Fig. 3 that the second of these gangliosides is the same as G0.5. Since the G0.5 has 33% neuraminidase-sensitive sialic acid while the mixture of the first and second components (*i.e.* 'slow') has 57% neuraminidase-sensitive sialic acid, the proportion of neuraminidase-sensitive sialic acid in the first component is probably greater than 57%. It is unlikely that the two metachromatic gangliosides are identical with the two gangliosides of Klenk & Gielen (1960) since these latter are 'fast' and non-metachromatic (Van Heyningen & Miller, 1961).

The work reported in this paper confirms the observation of Van Heyningen & Miller (1961) that tetanus toxin-fixation by ganglioside is dependent upon sialic acid content. Although not all the sialic acid of ganglioside is concerned in viral inhibition, it does all appear to be concerned in toxin-fixation. Thus, for example, G2.0, which has practically no viral inhibitory capacity and no neuraminidase-sensitive sialic acid, nevertheless fixes tetanus toxin in proportion to its sialic acid content. Similarly, when 'slow' ganglioside is treated with neuraminidase it retains its toxin fixing capacity in proportion to its residual sialic acid.

It is notable that there are sialic acid-containing substances having biological properties that are the reverse of those of the G2 type of ganglioside, for example, ovomucoid which is an effective inhibitor of viral haemagglutination but does not fix tetanus toxin (Van Heyningen & Miller, 1961).

The figures for the neuraminidase-sensitive sialic acid are subject to some variation and may not in every case represent maximal values, for the tendency of ganglioside to form aggregates may render them inaccessible to the enzyme bonds which would otherwise be attacked. There seem to be at least two possible explanations for differences in neuraminidase sensitivity of sialic acid in ganglioside: there may

be more than one kind of sialic acid linkage in ganglioside, or, the gangliosides themselves may form different types of micelles in which the availability of the bonds to the enzyme differs.

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