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ANTI-NEURAMINIDASE ANTIBODIES: WHICH SUBSTRATE SHOULD BE USED TO TEST THEIR EFFECT ON INFLUENZA VIRUS NEURAMINIDASE

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SUMMARY

The effect of two monoclonal anti-neuraminidase antibodies RANA 1 and WANA 1 on the enzymic activity of A/NWS$_{HA}$-Tokyo/3/67$_{NA}$ HON2 influenza virus has been demonstrated to differ according to the substrate. Three substrates were tested, a low molecular weight substrate N-acetylneuraminosyl lactose, the glycoprotein fetuin, and cultured human ALL T cells. The enzyme's activity was enhanced when the virus was complexed with either antibody and exposed to the cultured human lymphocytes. Furthermore, no inhibition was observed at a 20:1 ratio of antibody to enzyme. The enzymic activity was inhibited by both antibodies when the two non-physiological substrates were used.

1. INTRODUCTION

Neuraminidase, one of the two glycoproteins associated with the outer surface of the influenza virus (the other being haemagglutinin), hydrolyses the ketosidic linkage of terminal sialic acid (1-3). Anti-neuraminidase antibody has been shown to provide man with little or no immunity to the disease (4) and its function in the process of infection is not established. It has been suggested that the viral neuraminidase is involved with penetration of the virus into the host cell, release of progeny virus or in the prevention of aggregation of newly formed virus (5).

Recently, we have applied ^{1}H NMR spectroscopy to a study of the infectious process of the influenza virus (6,7). In an attempt to elucidate the role of neuraminidase, monoclonal anti-neuraminidase antibody was complexed with the influenza virus prior to infection. The antibody had been shown, by the Warren assay for sialic acid, to inhibit the activity of the enzyme when either N-acetylneuraminosyl lactose (NANLAC) (9) or the glycoprotein, fetuin, were used as substrates. The results were, however, not in accord with those with whole cells. The ^{1}H NMR experiments provided evidence that sialic acid could be hydrolysed from the cell surface of chicken embryo fibroblasts by this immune complex (8).

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Thus it appeard that the antibody did not inhibit the enzyme's activity against the substrate provided by a potential host cell.

As fetuin is commonly used to monitor the activity of influenza virus neuraminidase, these findings were extended. We report here the effect of two monoclonal anti-Tokyo/3/67-neuraminidase antibodies, RANA 1 and WANA 1, on the enzymic activity of A/NWS$_{HA}$-Tokyo/3/67$_{NA}$ HON2 influenza virus with two non-physiological substrates, NANLAC and fetuin, and with cultured human acute lymphoblastoid leukaemic T cells (CCRF/CEM).

2. MATERIALS AND METHODS

A/NWS$_{HA}$-Tokyo/3/67$_{NA}$ HON2 (T/67) influenza virus was grown as previously described (7).

The isolation of the two monoclomal anti-T/67 neuraminidase antibodies RANA 1 (Raison anti-neuraminidase antibody) and WANA 1 (Webster anti-neuraminidase antibody) was described previously (9,10,11).

400 HAU (12) of T/67 influenza virus was incubated with increasing concentrations of RANA 1 and WANA 1 for 3 hours at 37°. The virus alone and the immune complexes were added to tubes containing 1mM NANLAC (Boehringer Mannheim, Australia, Pty.Ltd.) or 70µM fetuin (Commonwealth Serum Laboratory, Australia) in 150 mM MES-NaOH, calcium-magnesium saline (0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.15M NaCl, 20 mM sodium borate, pH 6.5) in a total volume of 250µl. After incubation for three minutes at 30° in the case of NANLAC and 30 minutes for fetuin, the reaction was stopped by the addition of 100µl of periodate reagent (13).

Arsenite reagent (1.0 ml) (13) was added, and the tubes vortexed. Thiobarbituric acid (2.5 ml) (13) was added and the tubes placed in a boiling water bath for 15 minutes. The solution was cooled, the colour extracted with 10 ml of acidified butanol (50 ml of concentrated HCl in 1 litre of n-butanol) and left for 10 minutes. Under these conditions 150 nmoles sialic acid were equivalent to an absorbance of 0.34 at 549 nm in a 1.0 cm light path cuvette. (Where a large number of samples were involved they were stored at 4° before colour development). No colour was obtained in controls without substrate indicating that the enzyme was unable to cleave antibody bound sialic acid.

Human, acute lymphoblastoid leukaemic (ALL) T cells CCRF/CEM were grown in suspension culture in RPMI 1640 medium supplemented with 10% foetal calf serum. Cells, which were studied in the log phase of growth, were washed with saline prior to use.

4000 HAU of influenza virus was complexed with increasing concentrations of each antibody, added to 4 x 10^7 ALL T cells CCRF/CEM suspended in calcium-magnesium saline at pH 6.5 (as above) and incubated for one hour. The reaction was stopped and the assay procedure continued as described above. The colour was extracted into 2 ml of acidified butanol and read at 549 nm.

The ratio of antibody molecules to neuraminidase molecules was calculated assuming 200 neuraminidase molecules per virus particle, and 4 x 10^7 virus particles per HAU.

3. RESULTS AND DISCUSSION

The ability of anti-neuraminidase antibody to inhibit the enzyme activity of influenza virus neuraminidase is commonly tested by exposing the immune complex to the glycoprotein, fetuin. Our results suggested that such experiments may be misleading,

Both anti-neuraminidase antibodies (at an antibody:enzyme ratio of 1:1) partially inhibit the enzymic activity of T/67 influenza virus when incubated with NANLAC (Fig. 1A). WANA 1 continues to inhibit as the antibody levels are increased whereas RANA 1, at a 4:1 ratio causes 50% of the activity to be regained. The change in the enzymic activity of neuraminidase with different ratios of RANA 1 suggests that structural constraints are being imposed on the tetrameric enzyme which are overcome as more antibody molecules bind.

In the presence of 70μM fetuin a 50% enhancement of the enzyme's activity is observed after complexing to both antibodies (Fig. 1B). In contrast to the behaviour observed with NANLAC RANA 1 causes complete inhibition of the enzyme's activity at a 2:1 ratio when fetuin is the substrate.

400 HAU of uncomplexed T/67 influenza virus hydrolyses 8 nmoles of sialic acid from 4 x 10^7 ALL T cells per hour. After complexing with RANA 1 and WANA 1 the activity is enhanced by 30% (Fig. 1C). This enhancement remains despite increasing levels of antibody. At a ratio of 20:1 the activity of the immune complexes has decreased to just below that observed for the uncomplexed virus,

The drop in activity caused by RANA 1 at a 1:1 ratio with NANLAC is also observed with the whole cells (Fig. 1C).

The two monoclonal antibodies, RANA 1 and WANA 1 have previously been shown to recognise different antigenic determinants on the influenza virus neuraminidase (9), and be specific for different strains of post 1957 influenza virus (4). These data provide further evidence that RANA 1 and WANA 1 recognise different antigenic determinants on the neuraminidase molecule. The effect of increasing amounts of antibody on the enzymic activity of the influenza virus in the presence of a host cell differs from that observed with the two non-physiological substrates. The change in the neuraminidase activity caused by the anti-neuraminidase antibody is clearly dependent on the size and structure of the substrates.

Most important is the enhancement in the enzyme's activity which occurs upon complexing either anti-neuraminidase antibody in the presence of a potential host cell. When the antibody to virus ratio is sufficiently

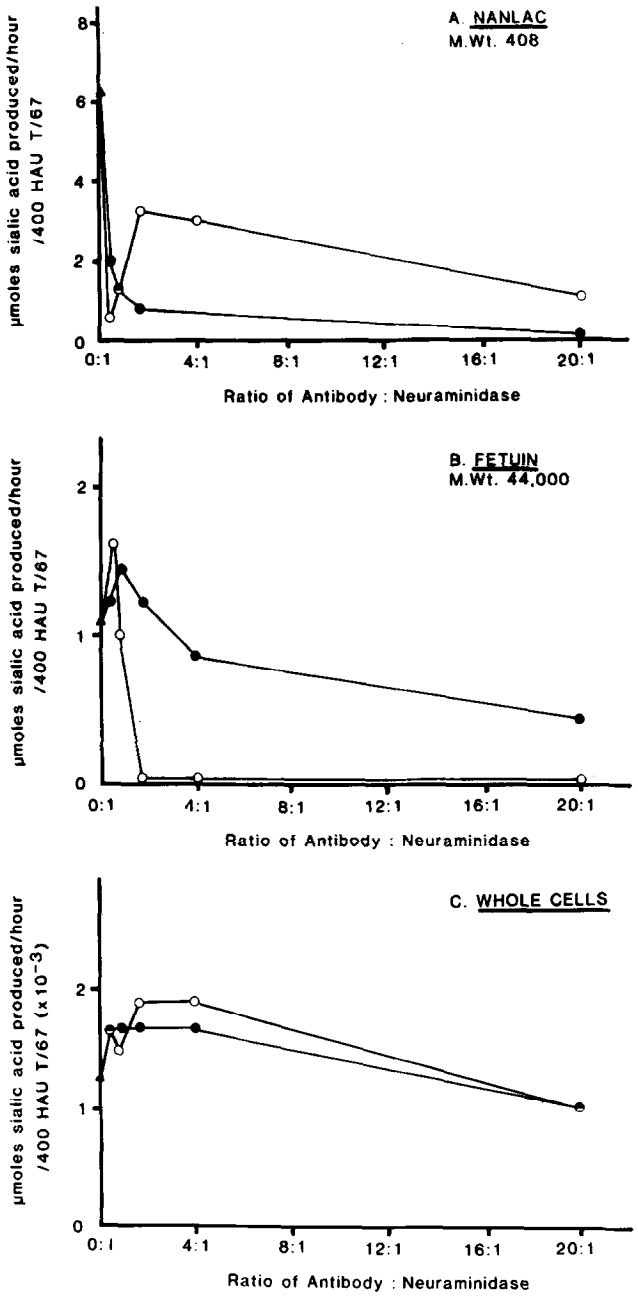

Figure 1

The amount of sialic acid liberated by the recombinant influenza virus A/NWS$_{HA}$-Tokyo/3/67$_{NA}$ HON2 when complexed with increasing amounts of the anti-neuraminidase antibodies RANA 1 and WANA 1. Free sialic acid levels were measured after virus and immune complexes were incubated with the following substrates: A, NANLAC (1 mM) B, Fetuin (70µM) C, Cultured human ALL T-cells, CCRF/CEM (4 x 10^7 cells).

Δ T/67 virus alone. ● T/67 complexed with WANA 1. ○ T/67 complexed with RANA 1.

high for a large immune complex to have formed the activity starts to decrease probably due to restricted access to the cell surface caused by the size of the complex. These results, therefore, suggest that the host cell has terminal sialic acid on the surface which is in an environment accessible to the immune complex.

The physiological implication of these results is that anti-neuraminidase antibody is indeed capable of preventing infection by influenza virus but only if present in high concentration in the serum. The ability of the immune response to remove these complexes in the presence of sialic acid is discussed at length elsewhere (14).

These data provide an explanation for the clinical trials reported by Yamane et al (6). where only the patients with four times the normal serum level of anti-neuraminidase antibody were immune to the new strain of influenza virus. We would suggest that in the presence of high titres of anti-neuraminidase antibody the neuraminidase activity was indeed inhibited and thus infection prevented.

4. CONCLUSIONS

The purpose of these experiments was to deduce which substrates could be used to provide biologically relevant information on the effect of anti-neuraminidase antibody on the infectious processes of the influenza virus.

The data presented here clearly demonstrate that neither NANLAC nor fetuin can be necessarily regarded as reliable substrates for deducing the biological role of influenza virus neuraminidase in either infection or immunity.

Enhancement of the enzyme's activity caused by these two anti-neuraminidase antibody at low concentration in the presence of a host cell could explain why people with normal titres of anti-neuraminidase antibody do not have immunity to the disease. These data which show inhibition of the enzymic activity to commence at high antibody to enzyme ratios are consistent with the clinical report that only patients with four times the normal serum levels of anti-neuraminidase antibody were immune to the disease (6).

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