Sialoglycoproteins that bind influenza A virus and resist viral neuraminidase in different animal sera

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Sialoglycoproteins that are resistant to degradation by viral neuraminidase can effectively neutralize influenza A viruses, because they bind irreversibly to the viruses. To detect such proteins in animal sera, we developed an immunochemical assay based on Western blotting techniques. We assessed the binding activity of sialoglycoproteins in sera from nine different animals toward the A/Aichi/2/68 (H3N2) and A/PR/8/34 (H1N1) strains of influenza virus, with or without viral and bacterial neuraminidase treatment. Using this assay, we found that animal sera contain a spectrum of sialoglycoproteins defined by differing abilities to bind influenza A viruses and to resist the viral neuraminidase. Structural analysis of these inhibitors would provide useful information for the development of anti-influenza virus compounds.

Animal sera contain three classes of non-specific inhibitors of influenza virus which can be distinguished by their biochemical properties. The α and γ classes of inhibitors are heat-stable glycoproteins that contain sialyloligosaccharides similar to those on cell surface receptors (Gottschalk et al., 1972). The γ but not α inhibitors can neutralize virus infectivity. The γ inhibitors, identified as α₂-macroglobulins in horse and guinea-pig sera, are potent inhibitors of H2N2 and H3N2 influenza viruses, most likely because of the presence of 4-O-acetyl-5-N-acetylneuraminic acid chains that resist hydrolysis by viral neuraminidase (Pritchett & Paulson, 1989). In addition to the γ inhibitors identified as the α₂-macroglobulins (Pritchett & Paulson, 1989; Ryan-Poirier & Kawaoka, 1993), other forms of γ inhibitory activity have been found in animal sera (Gottschalk et al., 1972), but their properties remain poorly defined.

Influenza viruses differ in their recognition of types of sialic acids and sialic acid–galactose (Gal) linkages (Rogers et al., 1986). A/PR/8/34 (H1N1) preferentially recognizes the Neu5Ac–α-2,3-DGal linkage in sialo-sugar chains over Neu5Ac–α-2,6Gal linkage, whereas A/Aichi/2/68 (H3N2) preferentially recognizes the Neu5Ac–α-2,6Gal linkage (Suzuki et al., 1986, 1992). By contrast, the neuraminidase that aids the release of virus from infected cells preferentially hydrolyses substrates with a Neu5Ac–α-2,3Gal linkage in sialo-sugar chains instead of the Neu5Ac–α-2,6Gal linkage (Suzuki et al., 1993). Because both the haemagglutinin and neuraminidase react with sialic acids, interplay between the two proteins in the oligosaccharide recognition must be important in the sensitivity of the virus to inhibitors.

In the present study, we searched for sialoglycoproteins capable of binding influenza virus while resisting hydrolysis by the viral neuraminidase, by developing an immunochemical assay based on Western blotting techniques.

Nine samples of animal sera were diluted 20-fold with SDS–PAGE sample buffer, and glycoproteins (0.25 to 2.5 μg) were directly solubilized in the same buffer. The respective preparations were separated on 4 to 20% SDS–polyacrylamide gradient gels under non-reducing conditions according to Laemmli (1970). They were then transferred onto polyvinylidene difluoride (PVDF) sheets (Nippon Millipore Kogyo) at a constant current of 1 mA/cm² for 80 min. The PVDF sheet was blocked with 5% BSA in PBS pH 7.2 containing 131 mM-NaCl, 14 mM-Na2HPO4, 1.5 mM-KH2PO4, 2.7 mM-KCl (reagent 1) at 37 °C for 3 h. After removal of the blocking solution, the sheet was incubated with purified influenza A virus [A/Aichi/2/68 (H3N2) and A/PR/8/34 (H1N1)] suspended in PBS at the concentration of 2⁶ to 2¹³ haemagglutination units (HAU) for 12 h at 4 °C, with subsequent incubation...
for 12 h at 37 °C. After removal of the viral suspension, the sheet was incubated with a solution of anti-haemagglutinin mouse monoclonal antibody (ascites diluted (0·1 ml/cm²) diluted 1000-fold with PBS containing 0·25% BSA (reagent 2) for 3 h, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG + IgM (H + L) (Jackson Immunoresearch Laboratories) diluted 1000-fold with reagent 2. Each step of the reaction was followed by washing with PBS five times. Virus--inhibitor binding was detected by immunostaining (HRP Kit IS-50B; Konica).

To establish optimal conditions for sialoglycoprotein binding to human influenza A virus, we tested a variety of parameters. At 4 °C, the binding of fetuin (2·5 μg; Gibco) to A/PR/8/34 increased linearly until 6 h of incubation with a concentration of virus (up to 211 HAU), after which the reaction was saturated (Fig. 1a, b). Glycophorin (Sigma) showed similar binding characteristics when tested with A/PR/8/34 at 4 °C in a corresponding assay (not shown). Linear increases in A/PR/8/34 binding were also detected over a range of fetuin concentrations (0·25 to 2·5 μg) (Fig. 1c). Treatment of fetuin and glycophorin with 30 mM-sodium periodate at 37 °C for 1 h or Arthrobacter ureafaciens neuraminidase (10 mU/ml; 0·5 ml/cm²; Nacalai Tesque) at 37 °C for 15 h before adding virus to the PVDF sheets completely abolished reactivity between A/PR/8/34 and the two sialoglycoproteins, indicating that the virus bound the sialoglycoproteins specifically (Fig. 1d).

To determine whether the viral neuraminidase can release virus bound on sialoglycoproteins, we examined the binding of A/PR/8/34 to glycophorin (2·5 μg) transferred onto PVDF sheets after preincubation with 211 HAU of A/PR/8/34 for 12 h at 4 °C and subsequent incubation for 24 h at 37 °C in the presence or absence of 1 mM-2-deoxy-2,3-dehydro-N-acetylneuraminic acid, a viral neuraminidase inhibitor (Meindl et al., 1974). In the absence of the neuraminidase inhibitor, the binding of A/PR/8/34 to glycophorin decreased as incubation time

Fig. 1. Validation of sialoglycoprotein--influenza A virus binding assay. A variety of parameters for sialoglycoprotein--influenza A virus binding (a, b and c) and specificity of the binding (d) was tested. Bovine fetuin was transferred onto PVDF sheets after electrophoresis. (a). Incubation of A/PR/8/34 at different times at 4 °C. (b) Incubation of different concentrations of A/PR/8/34 for 12 h. (c) Incubation of different concentrations of bovine fetuin on PVDF sheets with A/PR/8/34 for 12 h at 4 °C. (d) Bovine fetuin (lanes 1, 3, 5 and 7) and human glycophorin (lanes 2, 4, 6 and 8) were transferred onto PVDF sheets after electrophoresis. The sheets were treated with PBS (lanes 1 to 4), bacterial neuraminidase (lanes 5 and 6) or sodium periodate (lanes 7 and 8), and were then incubated with A/PR/8/34 for 12 h at 4 °C. A sheet incubated without virus (lanes 1 and 2) served as the negative control. A/PR/8/34 binding was detected by immunostaining and scanning.
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 increased and stopped entirely by 24 h at 37 °C (Fig. 2, lanes 1 to 4). However in the presence of the viral neuraminidase inhibitor, the virus remained bound even after incubation for 24 h at 37 °C (Fig. 2, lane 5). Under this condition, the two bands which are present above the intense band are a part of glycopherin (Furthmayr, 1978) as shown in Fig. 1(d), lane 4. These data indicate that viral neuraminidase can release virus bound to sialoglycoproteins on the PVDF membranes by removing the terminal sialic acid of the protein under the conditions used.

Before examination of influenza A virus binding, the presence of sialic acid and its linkage to terminal sugars in sialoglycoproteins of animal sera were characterized with biotin-labelled lectins. **Sambucus sieboldiana** agglutinin (SSA), which interacts with high affinity to the Neu5Ac-a-2,6Gal/GalNAc linkage (Shibuya et al., 1989), bound to a spectrum of sialoglycoproteins in all sera (data not shown). In comparison with the binding of SSA, *Maakia amurensis* mitogen (MAM), which interacts with high affinity to complex-type tri- and tetra-antennary Asn-linked oligosaccharides containing the Neu5Ac-a-2,3Gal linkage (Wang & Cummings, 1988), bound to only some of the glycoproteins in guinea-pig serum and to a few of those in other animal sera (data not shown). These lectins did not bind to proteins pretreated with bacterial neuraminidase, indicating that the lectin binding is specific. Treatment of carbohydrate with mild alkali (0.1 M-NaOH at 37 °C for 30 min) as previously described (Pritchett & Paulson, 1989) is routinely used to examine the role of alkali-labile groups (e.g. the O-acetyl group) in sugar moieties. Such treatment had no effect on the binding (not shown), indicating that alkali-labile groups on the carbohydrate sidechain are not important for recognition by the lectins and that the treatment did not alter the basic structure of the carbohydrate.

To detect potentially neutralizing serum sialoglycoproteins, we examined influenza A virus binding to sialoglycoproteins in nine different animal sera (human, mouse, rat, rabbit, equine, guinea-pig, goat, chicken and bovine), using the same assay system described in the preceding section. To be effective neutralizers of virus, serum sialoglycoproteins should not be efficiently hydrolysed by viral neuraminidase, thus permitting irreversible binding between the virus and the proteins. We therefore examined virus binding in the presence (37 °C) or absence (4 °C) of viral neuraminidase activity. A/Aichi/2/68 (H3N2), which preferentially recognizes the Neu5Ac-a-2,6Gal linkage in sialo-sugar chains more readily than the Neu5Ac-a-2,3Gal linkage (Shibuya et al., 1986; Rogers & D’Souza, 1989), bound to a spectrum of sialoglycoproteins in all sera at 4 °C (Fig. 3a). The binding pattern of A/Aichi/2/68 to the nine different animal sera was similar to that of SSA lectin which specifically interacts with the Neu5Ac-a-2,6Gal/GalNAc linkage (Shibuya et al., 1989). With an increase in temperature to 37 °C, most of the virus bound to sialoglycoproteins at 4 °C was not removed by viral neuraminidase treatment (Fig. 3b). As the A/Aichi/2/68 haemagglutinin binds preferentially to Neu5Ac-a-2,6Gal, the lack of influence of the viral neuraminidase on binding activity at 37 °C can be attributed to its preferential cleavage of Neu5Ac-a-2,3Gal rather than Neu5Ac-a-2,6Gal (Baum & Paulson, 1991). In the absence of virus, bands corresponding only to immuno-globulins were apparent, indicating low non-specific reactions (Fig. 3c).

![Fig. 2. Effect of viral neuraminidase on the binding of influenza virus to sialoglycoprotein. Glycophorin, transferred onto PVDF sheets after electrophoresis, was incubated with A/PR/8/34 for 12 h at 4 °C, followed by incubation at 37 °C for 3 h (lane 1), 6 h (lane 2), 12 h (lane 3) and 24 h (lanes 4 and 5) in the presence (lane 5) or absence (lanes 1 to 4) of 1 mM-2-deoxy-2,3-dehydro-N-acetylneuraminic acid. A/PR/8/34 binding was detected by immunostaining.](image_url)
Fig. 3. Sialoglycoproteins that bind to purified human influenza virus A/Aichi/2/68 (H3N2) and resist viral neuraminidase in animal sera. The nine animal sera (lanes 1 to 9) were transferred onto a PVDF sheet after SDS-PAGE and studied under various conditions. (a) Incubation of A/Aichi/2/68 for 12 h at 4 °C. (b) Incubation A/Aichi/2/68 for 12 h at 4 °C, followed by incubation at 37 °C for 12 h. (c) Treatment with bacterial neuraminidase, followed by incubation of A/Aichi/2/68. (d) Treatment with 0.1 M-NaOH, followed by digestion of bacterial neuraminidase and incubation with A/Aichi/2/68. (e) Incubation without virus (control). The Mr values of serum sialoglycoproteins were determined with an electrophoresis calibration kit (Pharmacia LKB). Sources of sera were: human (lanes 1), murine (lanes 2), rat (lanes 3), rabbit (lanes 4), equine (lanes 5), guinea-pig (lanes 6), goat (lanes 7), chicken (lanes 8) and bovine (lanes 9).

linkage (Wang & Cummings, 1988) may differ in their recognition of sialo-oligosaccharides. At 37 °C, the majority of the proteins (human, guinea-pig, goat and bovine sera) did not bind to A/PR/8/34, presumably owing to loss of the terminal sialic acids hydrolysed by the viral neuraminidase. Reduced binding to A/PR/8/34 at 37 °C would be expected from the preference of both haemagglutinin and neuraminidase for Neu5Ac-α,2-3Gal (Suzuki et al., 1986, 1992, 1993). However, proteins with apparent Mr values of 120K (guinea-pig serum) and 80K to 90K (mouse, rat, rabbit, equine, chicken and bovine sera) remained bound to the virus under this condition (Fig. 4b). That A/Aichi/2/68 bound to many sialoglycoproteins in all sera and most of the virus bound was not removed by viral neuraminidase indicates the possibility of neutralization of A/Aichi/2/68 infection by these sialoglycoproteins.

Treatment with A. ureafaciens neuraminidase, which can hydrolyse the common types of sialoglycosidic linkage except 4-O-acetylated sialic acids (Corfield et al., 1986), abolished the binding of A/Aichi/2/68 and A/PR/8/34 to sialoglycoproteins in animal sera (Fig. 3c and 4c). The exceptions to this were reactions involving A/Aichi/2/68 and 80K to 90K proteins found in all animal sera and many of those in equine and guinea-pig sera, which provided resistance to bacterial neuraminidase digestion (Fig. 3c). However the binding of A/Aichi/2/68 to sialoglycoproteins, was completely abolished by mild alkali treatment and subsequent bacterial neuraminidase digestion of the proteins (Fig. 3d), indicating that sialoglycoproteins that resisted the bacterial neuraminidase contain alkali-labile groups and that A/Aichi/2/68 binds to them. The alkali-labile sialoglycoproteins with affinity for A/Aichi/2/68 may
contain neuraminidase-resistant 4-O-acetyl-N-acetyl-
neuraminic acid like that of equine and guinea-pig α₂-
macroglobulin (Pritchett & Paulson, 1989). In contrast
to binding by A/Aichi/2/68, A/PR/8/34 binding to the
sialoglycoproteins was completely absent with only
bacterial neuraminidase digestion (Fig. 4c), indicating
that this virus does not bind sialoglycoproteins with
alkali-labile groups and that these proteins differ from
equine and guinea-pig α₂-macroglobulin. H3N2 human
viruses (e.g. A/Aichi/2/68) with leucine at position 223
of the haemagglutinin can bind sialic acids containing
4-O-acetyl-N-acetylneuraminic acid, whereas A/PR/8/34
cannot (Nobusawa et al., 1991; Matrosovich et al.,
1992). The neutralizing activity of α₂-macroglobulin is
presumably due to the large size of the protein, the
spatial arrangement of the sialo-oligosaccharide groups
and the presence of 4-O-acetyl-N-acetylneuraminic acid
(Pritchett & Paulson, 1989).

The pathogenicity of influenza virus in hosts such as
tissue cultures and animals may be affected by the serum
or body fluid sialoglycoproteins that bind to the virus but
are resistant to the viral neuraminidase, and finally
neutralize the virus. The assay system described in this
report allows screening for such sialoglycoproteins.

We thank John Gilbert for editorial assistance and Dayna Anderson
for typing the manuscript. This work was supported in part by grants-
in-aid 63480493 (Y. S.), 1880021 (Y. S.) and 01308028 (Y. S. and A.H.)
from the Ministry of Education, Science and Culture of Japan and a
grant-in-aid for scientific research from the Shizuoka Prefectural
Foundation for the Advancement of Education, Japan (Y. S.). Support
was also provided by Public Health Service research grant AI-20591
(R.G.W.) and AI-29599 (Y. K.) from the National Institute of Allergy
and Infectious Diseases, Cancer Center Support (CORE) grant CA-
21765 (R.G.W. and Y.K.), and the American Lebanese Syrian

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(Received 2 November 1993: Accepted 17 January 1994)