Basis for the Potent Inhibition of Influenza Virus Infection by Equine and Guinea Pig $\alpha_2$-Macroglobulin*

Thomas J. Pritchett and James C. Paulson

From the Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, California 90024

(Received for publication, November 21, 1988)

The unique properties of equine and guinea pig sera which make them potent inhibitors of influenza virus adsorption and infection have been investigated. The inhibitory activities of both sera are found to reside entirely in their respective $\alpha_2$-macroglobulins, high molecular weight glycoproteins which bind to viral hemagglutinins via sialic acids of their N-linked carbohydrate groups. Structure analysis has shown that both proteins contain 4-O-acetyl-N-acetylenuraminic acid (4-O-Ac-NeuAc) (Hanaoka, K., Pritchett, T. J., Takasaki, S., Kobata, T., 1989 J. Biol. Chem. 264, 9842–9849). These 4-O-acetylated sialic acids have been found in few species, making their coincidence with high inhibitory potency in equine and guinea pig $\alpha_2$-macroglobulin striking. However, 4-O-Ac-NeuAc does not appear to increase the avidity of interaction with influenza viruses since isolated oligosaccharides of equine $\alpha_2$-macroglobulin are no more potent inhibitors of adsorption than isolated oligosaccharides of human $\alpha_2$-macroglobulin, which is a relatively poor inhibitor and contains only NeuAc. Since 4-O-Ac-NeuAc is resistant to cleavage by virai sialidase it may serve to protect the inhibitor from inactivation. These and supporting results suggest that the key property of equine and guinea pig $\alpha_2$-macroglobulin which make them high potency inhibitors is a spatial arrangement of sialic acid containing oligosaccharide groups which allows optimal interaction with multiple hemagglutinins. The implications of these results for the design of low molecular weight inhibitors of influenza virus infection are discussed.

Sialic acid has been known as an essential receptor determinant of influenza viruses for over 40 years (reviewed in Refs. 1 and 2). Two viral envelope glycoproteins interact with sialic acid containing receptors. These are the hemagglutinin, which is the cell attachment protein, and the sialidase, which presumably aids in the elution of virus from the infected cell and in the destruction of sialic acid containing mucous glycoproteins that can act as receptor analogs and inhibit infection (1, 2).

Through analysis of the three-dimensional structure of the hemagglutinin complexed with a receptor analog, sialyllactose, Weiss et al. (3) have recently shown that sialic acid fills the receptor binding pocket of the hemagglutinin. Yet inhibi-...
high inhibitory potency is striking since this sialic acid has been found in few species (22) and has not previously been reported in guinea pig. However, as described here, 4-O-Ac-NeuAc does not appear to play a direct role in the high inhibitory potency of the equine and guinea pig $\alpha_2$-macroglobulins. Indeed, despite the difference in the inhibitory potency of the equine and human $\alpha_2$-macroglobulins, their isolated N-linked carbohydrate groups have equal potency for inhibition of influenza virus adsorption to erythrocytes, as do free NeuAc and 4-O-Ac-NeuAc. In contrast, on a sialic acid basis, equine $\alpha_2$-macroglobulin is a 4,000,000-fold more potent inhibitor than its free N-linked oligosaccharides.

The results suggest that the overriding factor in the inhibitory properties of animal $\alpha_2$-macroglobulins is their high valency, coupled with a spatial arrangement which allows optimal interaction with multiple influenza virus hemagglutinins. The significance of the coincidental occurrence of the rare sialic acid 4-O-Ac-NeuAc on the $\alpha_2$-macroglobulins of unusually high inhibitory potency may be related to its inhibition of the viral sialidase activity.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Choice of Sera**—Our initial goal was to identify the basis for the potent inhibitory properties of several animal sera, by purifying several "active" glycoproteins and compare their structures with corresponding glycoproteins from "low potency" sera. Of animal sera examined to date, equine, guinea pig, and hedgehog sera have been found to exhibit the highest inhibitory potency for influenza virus adsorption to cells (12, 14, 23). To determine the degree to which high inhibitory potency was unique in the animal kingdom, 47 sera from mammals, birds, a reptile, and fish were screened for the ability to inhibit hemagglutination by a human influenza virus, A/Memphis/102/72 (H3N2). While most sera surveyed possessed low inhibitory activity, potency varied widely within each order and family (Table 1; see miniprint supplement). The highest inhibitory potency was found with sera from horse, guinea pig, East African bongo, and the fish Heterodontis Francisci, which gave HAI titers of 1024-4096. These results indicate that potent HAI activity is a property exhibited by the sera of relatively few species.

For further characterization equine and guinea pig sera were chosen as high potency sera because of their ready availability. Human serum was chosen as a low potency inhibitor for comparison. Equine and human sera have additional biological significance, since horses and humans are both natural hosts of influenza.

**$\alpha_2$-Macroglobulin Is the Only High Potency Inhibitor in Horse Plasma**—Several investigations have reported that purified equine $\alpha_2$-macroglobulin is a potent inhibitor of human influenza virus attachment to cells, an activity mediated by its sialic acid containing carbohydrate groups (15, 19). Since serum glycoproteins may carry similar carbohydrate structures it was important to ascertain whether or not the inhibitory activity of equine sera was due to several glycoproteins or $\alpha_2$-macroglobulin alone. Accordingly, the inhibitory activity of equine plasma was followed by inhibition of hemagglutination by A/Memphis/102/72, during purification of $\alpha_2$-macroglobulin.

Purification was achieved with a two-step procedure involving Cibacon Blue Sepharose 6B chromatography (24) followed by zinc chelate affinity chromatography (25, 26). Essentially all of the hemagglutination inhibition (HAI) activity (Fig. 1, vertical bars) coeluted with $\alpha_2$-macroglobulin during Cibacon Blue Sepharose chromatography, which removed the majority of plasma proteins (Fig. 1). With further purification of the $\alpha_2$-macroglobulin by zinc chelate affinity chromatography, all the HAI activity bound and eluted with $\alpha_2$-macroglobulin (Fig. 2). This method resulted in an $\alpha_2$-macroglobulin preparation in which 90-95% of the total protein migrated as a single band with an apparent molecular weight of 180,000 on SDS-PAGE under reducing conditions (Fig. 2, insert, lane 7) and which contained virtually all the HAI activity (Fig. 2, vertical bars). The results show that $\alpha_2$-macroglobulin is the only potent glycoprotein inhibitor of influenza virus present in horse plasma.

**Purification of Guinea Pig and Human $\alpha_2$-Macroglobulin**—Using essentially the same procedure, $\alpha_2$-macroglobulin could also be purified from guinea pig serum and human plasma. Analysis of purified guinea pig $\alpha_2$-macroglobulin by SDS-PAGE showed characteristic properties of $\alpha_2$-macroglobulins purified from serum (Fig. 3a) (27). Without reduction (lane 3) it appeared as two closely spaced major bands with relative molecular weights ($M_r$) greater than that of myosin heavy chain. With reduction (lane 2), the majority of the protein appeared as two bands with $M_r$ of 180,000 and 166,000 corresponding, respectively, to the electrophoretically fast form (the only form seen when $\alpha_2$-macroglobulin is isolated from plasma) and the electrophoretically slow form generated when $\alpha_2$-macroglobulin reacts with active proteases released when blood is clotted to obtain serum (27). Several bands of lower $M_r$ are probably degradation products (27) since these bands were absent when the sample was run without reduction (lane 3). In this regard, a similar preparation of equine $\alpha_2$-macroglobulin was obtained when isolated from horse serum rather than horse plasma (not shown).

Purification of $\alpha_2$-macroglobulin from human plasma yielded a preparation similar to that obtained from equine plasma. Analysis by SDS-PAGE under reducing conditions

**FIG. 1. Purification of equine $\alpha_2$-macroglobulin by Cibacon Blue Sepharose chromatography.** As described under "Experimental Procedures," dialyzed horse plasma (25 ml) was chromatographed on a column (2.5 x 80 cm) containing 400 ml of Cibacon Blue Sepharose. Fractions (1 ml) were assayed for absorbance at 280 nm (○), trypsin esterase activity (■, $\alpha_2$-macroglobulin), sialic acid (□), and hemagglutination inhibition activity (HAI) (vertical solid bars), then pooled as indicated by the horizontal black bar.
Fig. 2. Purification of equine α2-macroglobulin by Zn²⁺ affinity chromatography. Pooled α2-macroglobulin containing fractions from Cibacron Blue chromatography (horizontal bar, Fig. 1) were loaded onto a column (3.3 × 36 cm) containing 100 ml of chelating Sepharose 6B (Pharmacia) charged with zinc ions. The column was washed at pH 6.0 until a stable base line of absorbance at 280 nm was observed, and then α2-macroglobulin was eluted by changing the buffer pH to 5.0 (arrow). Fractions (8 ml) from the wash and elution steps were monitored for absorbance at 280 nm (0) and assayed for hemagglutination inhibition activity (vertical bars), then pooled as indicated by the horizontal bar. Inset shows nonreduced (1-3) and reduced (5-7) SDS gel electrophoresis of horse plasma (I and 5), pooled Cibacron Blue fractions (2 and 6), and pooled fractions eluted from Zn²⁺ chelate column (3 and 7). Lane 4, M, markers (Sigma high molecular weight mixture): myosin, β-galactosidase, phosphorylase b, bovine albumin, egg albumin (top to bottom). Details of the electrophoresis procedure are given in the legend to Fig. 3.

Fig. 3. Analysis of purified human and guinea pig α2-macroglobulin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A, electrophoretogram of guinea pig α2-macroglobulin. Lane 1, relative molecular weight (Mr) standards; lane 2, sample run under reducing conditions; lane 3, nonreduced sample. B, electrophoretogram of human α2-macroglobulin. Lane 1, sample run under reducing conditions; lane 2, M, standards. Discontinuous SDS-PAGE was performed essentially as previously described (52). A 4% (w/v) acrylamide stacking gel and a 7% resolving gel were used. Protein samples (10 μg) were heated at 100 °C for 3 min in sample buffer containing (final concentrations) 10% (v/v) glycerol and 2% (w/v) SDS (nonreduced) or glycerol and SDS plus 2.5% 2-mercaptoethanol (reduced) before being loaded onto the gel. The protein bands were visualized by staining with Coomassie Brilliant Blue R (Sigma).

revealed that greater than 95% of the protein was in a single band with Mr of 173,000 (Fig. 3B, lane 1).

Inhibitory Activity of Purified α2-Macroglobulins—As observed in the purification of equine α2-macroglobulin, essentially all of the HAI activity of guinea pig serum and most of the HAI activity in human plasma copurified with α2-macroglobulin. This can be seen in Table II which compares the HAI activity of plasma (or serum) with that of purified α2-macroglobulin at 3 mg/ml, the reported concentration in serum (28). The activity of equine and guinea α2-macroglobulin accounted for the very high potency of their sera, giving titers of 4096 and 2048, respectively. Human α2-macroglobulin was much lower in potency, with an HAI titer of 64. In all three cases, however, the inhibitory titer of the purified α2-macroglobulin was equal to or greater than that obtained using the corresponding serum. The purified α2-macroglobulins from two other low potency sera, bovine and chicken, also exhibited very low inhibitory potency.

Table II
Comparison of the inhibition of hemagglutination by serum and purified α2-macroglobulins

| Species   | HAI titer* | α2M |
|-----------|------------|-----|
| Serum     |            |     |
| Equine    | 4096       |     |
| Guinea pig| 2048       |     |
| Human     | 32         | 64  |
| Bovine    | 32         | 2   |
| Chicken   | 8          | 2   |

* HAI titer is expressed as the reciprocal of the highest dilution of the test substance causing inhibition of native human erythrocyte agglutination by 4 hemagglutinating units of A/Memphis/102/72.
Neutralization of Infection by Purified α2-Macroglobulins—Potent serum inhibitors of influenza viruses have been reported to be potent inhibitors of infection (14, 15). To verify the ability of purified α2-macroglobulins to neutralize viral infectivity, influenza A/Memphis/102/72 (H3N2) was exposed to increasing concentrations of human, equine, and guinea pig α2-macroglobulin just prior to adsorption to Madin-Darby canine kidney cells. As shown in Fig. 4, equine and guinea pig α2-macroglobulins were powerful inhibitors of viral infection, causing 50% inhibition of plaque formation at concentrations of 0.04 and 0.18 μg/ml, respectively. Human α2-macroglobulin was a much less potent inhibitor of infection, with 50% inhibition at 5.4 μg/ml, 30-120-fold greater than the equine and guinea pig α2-macroglobulins (Fig. 4).

Examination of the Role of 4-O-Acetyl-NeuAc in the Inhibitory Potencies of α2-Macroglobulins—Levinson et al. (19) provided early evidence that the inhibitory activity of equine α2-macroglobulin toward influenza isolates with the H2 hemagglutinin was dependent on the presence of 4-O-acetyl sialic acids. As described by Hanaoka et al. (21), both equine and guinea pig α2-macroglobulin contain 4-O-Ac-NeuAc as 30-50% of their total sialic acids, consistent with the possibility that 4-O-Ac-NeuAc is the common denominator in the potent inhibitory activity of the two proteins. To examine this point, the effects of periodate oxidation, mild base treatment, and digestion with Clostridium perfringens sialidase on the HAI activities of equine, guinea pig, and human α2-macroglobulin were monitored. The point of attack of each treatment on the structure of 4-O-Ac-NeuAc is illustrated in Fig. 5, and the results are summarized in Table III.

Very mild conditions of periodate oxidation selectively cleave adjacent hydroxyl groups of the polyhydroxyl side chain of sialic acid with the loss of one or two carbons (29-31). The complete abolition of HAI by periodate suggest that the inhibitory activity is dependent upon sialic acid and that the polyhydroxyl side chain is uninfected at the 8 and 9 positions (22). Subsequent reduction with sodium borohydride (29) restored inhibitory activity to approximately 20% of its original value, providing evidence that the hemagglutinin can bind to sialic acid with a shortened side chain terminated with a hydroxyl group, but not with a C-7(C8) aldehyde group.

The HA1 activities of equine and guinea pig α2-macroglobulin were essentially stable to sialidase digestion, showing only a 2-fold drop in each case (Table III). Colorimetric analysis revealed that 30 and 50% of total sialic acids remained glycosidically bound to equine and guinea pig α2-macroglobulin, respectively, consistent with the presence of sialidase-resistant 4-O-acetyl-NeuAc (32) on the N-linked carbohydrate groups of these proteins (21). However, if these two glycoproteins were first treated with mild base, which removes O-acetyl groups (Fig. 5; 32), the inhibitory activity was reduced only 4-8-fold, in repeated experiments (see Table III). Subsequent sialidase treatment quantitatively removed sialic acids and completely abolished the inhibitory activity of both the equine and guinea pig α2-macroglobulins.

Taken together these results show that sialic acids mediate the interaction of equine and guinea pig α2-macroglobulin with influenza virus. While their 4-O-Ac-NeuAc content alone is sufficient to account for their potent inhibitory activities (see activity after sialidase treatment, Table III), the two glycoproteins remain 16-32-fold more potent inhibitors than human α2-macroglobulin following removal of O-acetyl groups (see base treatment, Table III).

In contrast to the equine and guinea pig proteins, the low inhibitory activity of human α2-macroglobulin, which contains only NeuAc, was abolished by sialidase digestion prior to base treatment (Table III), and colorimetric analysis revealed that approximately 99% of the total sialic acids of human α2-macroglobulin had been removed.
Acids—Analysis of data in Table as a source of the inhibitory potency. To ascertain directly absence of GalNac in the sugar composition of these proteins

Drate Groups with Intact az-Macroglobulins and Free Sialic actually slightly more potent than those of equine az-macroglobulin (Fig. 6), despite the fact that the equine az-macroglobulin have similar size but differ 75-fold in inhibitory potency. a2-Acid glycoprotein and fetuin are also similar in size but differ 75-fold in inhibitory potency. This difference cannot be explained by the additional O-linked carbohydrate groups of fetuin, since sialylated anti-freeze glycoprotein derivatives with the identical sequences exhibited negligible inhibition at 100-fold higher concentrations.

**Relative Rates of Inactivation of Equine a2-Macroglobulin and De-O-Acetylated Equine a2-Macroglobulin by Viral Sialidase**—The presence of 4-O-Ac-NeuAc in both equine and guinea pig az-macroglobulin raises the question of its possible biological relevance. The 4-O-acetyl group is well known to confer resistance to digestion by a variety of bacterial and viral sialidases (19, 22). To determine if this was the case for a recent N2 sialidase, A/Memphis/102/72 (H3N2) was assessed for its ability to inactivate the inhibitory activity of az-Macroglobulin with and without 4-O-A-NeuAc (native and base-treated, respectively; Table IV). Native and base treated equine az-macroglobulin were incubated with concentrated virus and their inhibitory activities were monitored over a 24-h period, as shown in Fig. 7. The inhibitory activity of the base-treated glycoprotein was rapidly inactivated, with a half-time of inactivation of approximately 10–

| Inhibitor             | Concentration for 50% inhibition | Relative inhibitory potency |
|-----------------------|----------------------------------|-----------------------------|
| a-Methyl-NeuAc        | 2,000                            | 1.0                         |
| Human azM, oligosaccharides | 1,100                        | 1.8                         |
| Equine azM, oligosaccharides | 2,300                        | 0.9                         |
| NeuAc                 | 43,000                           | 0.047                       |
| 4-O-Acetyl-NeuAc      | 56,000                           | 0.036                       |
| Human azM             | 0.26                             | 7,700                       |
| Equine azM            | 0.00052                          | 3,800,000                   |

*Each glycoprotein, oligosaccharide, or free sialic acid was examined for its ability to inhibit A/Memphis/102/72 adsorption to resialylated erythrocytes modified to contain 18 nmol of NeuAc/ml packed cells NeuAc in the NeuAca2,6Gal linkage as described under “Experimental Procedures.”*

*Inhibitory potency is expressed relative to a-Methyl-NeuAc.*

*Isolation of N-linked oligosaccharides az-macroglobulin is described under “Experimental Procedures.”*

*NeuAc was from Sigma and 4-O-acetyl-NeuAc was isolated by H. Higa as described previously (7).*

*Native glycoprotein.*

**Comparison of Inhibitory Potencies of N-Linked Carbohydrate Groups with Intact az-Macroglobulins and Free Sialic Acids—**Analysis of data in Table III suggested that the equine and guinea pig az-macroglobulins retained most of their inhibitory potency after 4-O-acetyl groups were removed. The absence of GalNAc in the sugar composition of these proteins excludes sialic acid containing O-linked carbohydrate groups as a source of the inhibitory potency. To ascertain directly whether or not the N-linked carbohydrate groups of equine and human az-macroglobulins differed in their avidity for the H3 hemagglutinin, free carbohydrate groups were isolated following N-glycanase digestion. These were then compared for inhibition of viral adsorption using an assay developed for low molecular weight sialosides (20). As shown in Fig. 6, the isolated oligosaccharides of human az-macroglobulin were actually slightly more potent than those of equine az-macroglobulin (Fig. 6), despite the fact that the equine az-macroglobulin oligosaccharides retained the majority of their 4-O-acetyl groups during the release and isolation process, as determined by sialidase resistance (not shown). Using the concentration required for 50% inhibition as a basis of comparison, the simplest possible sialoside, a-methyl-NeuAc was comparable in inhibitory potency (Table IV). Moreover, the inhibitory potencies of free NeuAc and 4-O-Ac-NeuAc were essentially equal (only 5% the active a-anomer; 20). Taken together, these results shown that neither the 4-O-Ac-NeuAc-NeuAc substituent, or any other structural feature of the N-linked carbohydrate groups account for the high inhibitory potency of equine az-macroglobulin.

Also shown in Table IV for contrast are the inhibitory activities of the intact human and equine az-macroglobulins. These were 4,000–4,000,000 times more potent inhibitors, respectively, than were the oligosaccharides of these proteins (Table IV). The result emphasizes the importance of structural features contributed by polypeptide in the inhibitory properties such as valency, size, and the arrangement of the carbohydrate groups on the polypeptide backbone.

**Comparative Inhibitory Potencies of a Variety of Glycoproteins with Known Carbohydrate Structures—**Results presented thus far support the conclusion that the high inhibitory potencies of equine and guinea pig az-macroglobulin are not mediated by a unique feature of the carbohydrate moieties of these glycoproteins. Further evidence that inhibitory potency does not necessarily correlate with carbohydrate structure can be seen in a comparison of the relative abilities of several glycoproteins with known terminal carbohydrate sequences to inhibit hemagglutination by A/Memphis/102/72 as summarized in Table V (miniprint supplement).

Based on previous studies, A/Memphis/102/72 exhibits preferential binding to sialosides with the terminal SAa2,6Gal linkage (10). Yet, glycoproteins which contain the terminal sequence SAa2,6Gal21,4GlcNAc on N-linked oligosaccharides, differ in by 33,000-fold in their inhibitory potency. Molecular size may be an important factor, since among glycoproteins with similar carbohydrate groups, those with larger size appear to have higher inhibitory potency. However, there are notable exceptions. Human and equine az-macroglobulin have similar size, but as already demonstrated differ 40–100-fold in inhibitory potency. az-Acid glycoprotein and fetuin are also similar in size but differ 75-fold in inhibitory potency. This difference cannot be explained by the additional O-linked carbohydrate groups of fetuin, since sialylated antifreeze glycoprotein derivatives with the identical sequences exhibited negligible inhibition at 100-fold higher concentrations.

**TABLE IV**

Inhibition of A/Memphis/102/72 adsorption to erythrocytes by N-linked oligosaccharides, free sialic acids, and native human and equine az-macroglobulins

| Inhibitor                  | Concentration for 50% inhibition | Relative inhibitory potency |
|----------------------------|----------------------------------|-----------------------------|
| a-Methyl-NeuAc             | 2,000                            | 1.0                         |
| Human azM, oligosaccharides| 1,100                            | 1.8                         |
| Equine azM, oligosaccharides| 2,300                        | 0.9                         |
| NeuAc                      | 43,000                           | 0.047                       |
| 4-O-Acetyl-NeuAc           | 56,000                           | 0.036                       |
| Human azM                  | 0.26                             | 7,700                       |
| Equine azM                 | 0.00052                          | 3,800,000                   |

*No O-linked carbohydrate groups are present on equine, human, or guinea pig azM as judged by the lack of N-acetylgalactosamine during amino sugar analysis (T. Pritchett and J. Paulson, unpublished data).*
The high inhibitory potency exhibited by equine and guinea pig sera is found in relatively few species and can be accounted for entirely by a single glycoprotein, α2-macroglobulin. The inhibition of influenza virus adsorption to cells is mediated through the binding of the hemagglutinin to terminal sialic acids on N-linked, carbohydrate groups (Table III). However, there appears to be no unique feature of the carbohydrate structures of equine and guinea pig α2-macroglobulins which can account for their potent inhibitory properties. Although both of these glycoproteins contain 4-O-acetyl neuraminic acid, the 4-O-acetyl substitutions do not appear to be involved in binding affinity for the following reasons. 1) The free sialic acids, NeuAc and 4-O-Ac-NeuAc have approximately equal inhibition of influenza virus adsorption to erythrocytes. 2) Isolated N-linked carbohydrate groups from equine and human α2-macroglobulin, with and without 4-O-Ac-NeuAc, respectively, also have equal inhibitory potencies and are indistinguishable from the simple sialoside α2-methyl-NeuAc. 3) Crystal structure localization of sialic acid in the receptor binding pocket of the H3 hemagglutinin by Weiss et al. (3) shows that the 4-OH projects out of the pocket into solvent. Thus, there is no possibility for an O-acetyl substitution at that position to influence the binding interaction with the hemagglutinin.

The fact that equine α2-macroglobulin exhibits 4,000,000-fold higher inhibitory potency than the free N-linked oligosaccharides indicates the important role of the polypeptide chain. The importance of valency has been noted previously by Gottschalk et al. (16) and Fazekas de St. Groth and Gottschalk (33) and is supported by studies showing dramatic increases in inhibitory potency of glycoproteins following chemical cross-linking or aggregation (34, 35). However, human and equine α2-macroglobulins have similar size and number of sialic acids per mol, yet differ 50-100-fold in inhibitory potency. Thus, it appears that the major difference between these two glycoproteins reflects a more favorable distribution of the N-linked carbohydrate groups on the surface of the equine protein, allowing for a higher valency interaction with the hemagglutinins of the virus.

Although Pepper (17) and Levinson et al. (19) concluded that 4-O-Ac-NeuAc was required for the inhibitory activity of equine α2-macroglobulin against influenza H2 viruses, their data are largely consistent with the results reported here. Their conclusion was based on the sialidase resistance of the inhibitory activity and a 30-fold drop in inhibitory potency following mild base treatment. The results in Table III are similar except a smaller decrease in HAI was observed following base treatment in this report (4-8-fold). This drop is likely due to a base-mediated alteration in the conformation of the protein that disrupts the complementary contacts between the glycoprotein carbohydrate groups and the viral hemagglutinins.

The presence of α2-macroglobulins in pleural fluids (36) and their active secretion by alveolar macrophages (37) make them candidates as physiologically relevant inhibitors. In this regard, all equine H3 isolates examined to date (1963-1987) are 100-1000-fold less sensitive to inhibition of hemagglutination by horse serum than human influenza viruses (10, 15). It is well documented that equine α2-macroglobulins can mediate the selection of inhibitor resistant receptor variants from inhibitor sensitive H2 and H3 human influenza isolates (10, 12, 13, 15). Thus equine α2-macroglobulin or some other glycoprotein inhibitor could account for the selection and maintenance of the inhibitor insensitive phenotype of equine H3 influenza viruses.

The coincidence of finding 4-O-Ac-NeuAc in the two α2-macroglobulins that exhibit high inhibitory potency is striking. Prior to the report of Hanaoka et al. (21) which demonstrates 4-O-Ac-NeuAc in guinea pig α2-macroglobulin, this sialic acid had been found only in horses, donkeys, and the echidna, a monotreme (22). A report by Shortridge and Landsdell (29) suggests that this coincidence extends to inhibitors of hedgehog serum. Indeed, the inhibitor potency of hedgehog serum is similarly resistant to digestion by bacterial sialidase and destroyed by mild periodate treatment, hallmarks for the presence of 4-O-Ac-NeuAc (32). Although the 4-O-acetyl substituent does not influence the avidity of the interaction with the hemagglutinin, it is well known to inhibit bacterial and viral sialidase activity (22; Fig. 7). Thus, the 4-O-acetyl substitution would protect these α2-macroglobulins from inactivation by sialidase during infection. Because virulent infections are capable of killing large percentages of the populations of some species (38, 39), it is possible that glycoproteins with high inhibitory potency and 4-O-acetyl substituents to protect against inactivation could have arisen by coevolution as a protection against virulent influenza viruses.

With increased understanding of the receptor specificities of influenza viruses and the recent localization of sialic acid in the receptor binding pocket of the hemagglutinin crystal structure, attention has been focussed on the possibility for design of low molecular weight receptor analogs as inhibitors of infection by influenza virus (3, 20). We begun this work to identify structural features of the most potent natural inhibi-

5 J. Paulson, Y. Kawaoka, and R. Webster, unpublished results.
itors which might be useful in the design of such analogs. The results reveal that the single most important factor is not a unique structural feature of the carbohydrate sequence, but the effective valency of the interaction of the glycoprotein with the virus. Accordingly, rational drug design should include the synthesis and testing of multivalent sialoside analogs.

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**SUPPLEMENTAL MATERIAL TO BASE FOR POTENT INHIBITION OF INFLUENZA INFECTION of alpha-2-MACROGLOBULIN**

**EXPERIMENTAL PROCEDURE**

*Maniatis* - Common chicken inosine was purchased from Mallinckrodt, St. Louis, Mo. Equine antisera was from Fisher Biochemicals, Neponset, Mass. Purified chicken immunoglobulin was purchased from Sigma Chemical Company, St. Louis, Mo., and was used at a concentration of 5 mg/ml. In order to avoid possible problems with the injection site, the chicken immunoglobulins were not injected directly into the tissue culture medium.

**Supplemental Material**

**To:**

**Basis for Potent Inhibition of Influenza Infection by alpha-2-Macroglobulin**

Thomas J. Pritchett and James C. Paulson

**EXPERIMENTAL PROCEDURE**

**Chicken Blue Synagogue Cytopathic Changes** - After injection of the chicken blueSYNC virus, the animals were observed for 24 hours. A diagnosis of a typical large scale paralysis was made. Postmortem examination of the animals revealed that the chickens had died of influenza. The chickens were observed for 24 hours and then killed. The virus harvested from the chickens was used to infect a sensitive cell line, which was then observed for cytopathic changes.

**Purification of alpha-2-Macroglobulin**

*Chicken Blue Synagogue Cytopathic Changes* - A light yellow effluent was prepared from the infected chickens. The effluent was then clarified by centrifugation at 10,000 x g for 30 minutes. The clarified effluent was then filtered and used as the source of alpha-2-macroglobulin.

**Titration of alpha-2-Macroglobulin**

**Effect of alpha-2-Macroglobulin on Influenza Infection**

Thomas J. Pritchett and James C. Paulson

**EXPERIMENTAL PROCEDURE**

**Titration of alpha-2-Macroglobulin**

**Viral Specificity of alpha-2-Macroglobulin**

Quantitative assays for viral specificity were performed using a panel of alpha-2-macroglobulins. The assays were performed using a panel of alpha-2-macroglobulins which had been previously described. In each case, the virus titer was determined by plaque assay, and viral specificity was determined by the percentage reduction in plaque formation.

**Viral Specificity of alpha-2-Macroglobulin**

The results of the assays were expressed as the percentage reduction in plaque formation. The results are presented as the mean of three independent experiments. The viral specificity of alpha-2-macroglobulin was determined in a panel of alpha-2-macroglobulins which had been previously described. Each experiment was performed in triplicate, and the results were expressed as the percentage reduction in plaque formation compared to the control.

**Viral Specificity of alpha-2-Macroglobulin**

Step 1. Equin alpha-macroglobulin (44 mg) was titrated with 32000000 cpm of [125I]Esperoalpha (20 mg) by titrating the virus with alpha-2-macroglobulin (0.02 mg) prepared from the respective chicken and rabbit sera. The virus was then titrated with the respective alpha-2-macroglobulin.

**Quantitative assays for viral specificity**

Quantitative assays for viral specificity were performed using a panel of alpha-2-macroglobulins. The assays were performed using a panel of alpha-2-macroglobulins which had been previously described. In each case, the virus titer was determined by plaque assay, and viral specificity was determined by the percentage reduction in plaque formation.

**Viral Specificity of alpha-2-Macroglobulin**

Quantitative assays for viral specificity were performed using a panel of alpha-2-macroglobulins. The assays were performed using a panel of alpha-2-macroglobulins which had been previously described. In each case, the virus titer was determined by plaque assay, and viral specificity was determined by the percentage reduction in plaque formation.
Step 2. Two units of Peptide N-Glycosidase F was added, the solution was gently mixed, and incubated at 37°C. At 2 hour intervals an additional 2 units of enzyme was added up to a total of 8 units and 4 additions. Incubation was continued for a total of 18 hours.

Step 3. To isolate free oligosaccharides, the solution from step 2 was loaded directly onto a column (2.3 x 30 cm) consisting of 3.5 g Sepharose S-300. The column was eluted with 0.1 M ammonium bicarbonate at a flow rate of 50 ml per hour, and 2 ml fractions were collected. Oligosaccharides eluting from the column were detected by using 0.01 M o-nitroaniline per minute as the indicator. α2-Macroglobulin above, was pooled and concentrated by dialysis. Yield, on a unit of bulk, was 0.01.

Table I: Survey of Human Substances with α2-Macroglobulin Inhibitory Activity

| Species | Substances | Time | Inh. (%) |
|---------|------------|------|---------|
| Human   | Rabbit Anti-Rabbit | 24   | 100     |
| Monkey  | Human Anti-Monkey | 24   | 100     |
| Mammals | Human Anti-Mammals | 24   | 100     |

Table V: Inhibitory Potency of Glycopeptides Having Terminal α2-Macroglobulin Receptors

| Glycopeptide | Molecular Weight | Glycosyl Content | Terminal α2-Macroglobulin Receptor |
|--------------|------------------|------------------|------------------------------------|
| Galactosyl   | 4000             | 100              | 90                                 |
| O-Acetyl     | 4000             | 100              | 90                                 |
| Galactosyl   | 5000             | 100              | 90                                 |
| O-Acetyl     | 5000             | 100              | 90                                 |

α2-Macroglobulin Inhibitors of Influenza Virus

* Hemagglutination inhibition (HAI) was performed as described in Experimental Procedures. This test is exposed in the higher degree of serum causing inhibition of avian hemagglutination by 4 hemagglutination units of A/Amph/o/1/52.