On the Receptor of Influenza Viruses

1. Artificial Receptor for Influenza Virus

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Neuraminidase-treated erythrocytes coated with a variety of neuraminic acid-containing glycoproteins adsorbed influenza virus and neuraminidase-free hemagglutinins, but not purified neuraminidase. The results indicate that neuraminic acid is the binding site on the glycoprotein and that the rest of the molecule does not play a specific role in the reaction.

It is only for the myxoviruses that the chemistry of the cellular receptors has been elucidated, at least to some extent. From studies with soluble glycoproteins which can inhibit hemagglutination, it has been shown that the receptor site is *N*-acetylneuraminic acid (NANA), which is located on the terminal position of the oligosaccharide side chains. The soluble inhibitory glycoproteins and the cellular receptors are regarded as chemical analoges, competing for and serving as substrates for the neuraminidase of myxoviruses. Thus virus adsorption has been regarded as an enzyme-substrate linkage (for ref. see [1]). Recently this view has been revised, since it has been shown that myxoviruses could attach to cell surfaces even if virus neuraminidase had been inhibited by specific antibodies [2, 3], or other inhibitors [4, 5]. In addition, neuraminidase-free hemagglutinin has been isolated which adsorbs to red cells [6, 7], therefore, can be assumed, that both the neuraminidase and the hemagglutinin react with the cellular receptor.

Tiffany and Blough [8] recently demonstrated that fetuin-containing artificial membranes exclusively bind myxoviruses; the receptor function was lost, if NANA had been removed from the substrate. In order to elucidate further the functional significance of NANA, as the dominating compound of the myxovirus receptor, red blood cells were coated with NANA-containing glycoproteins of different structures and their virus binding capacities were tested. It was tried to define, whether neuraminidase or the hemagglutinating component of the virus surface are responsible for the attachment of influenza viruses.

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Material and Methods

Viruses. The following viruses were used: Fowl plague virus (FPV), strain "Rostock", influenza A/PR8, influenza A2/Asia, virus N, Newcastle disease virus (NDV), strains "Italien" and "Beaudette", parainfluenza I (Sendai), Sindbis virus, and vesicular stomatitis virus (VSV).

Soluble hemagglutinin with no measurable amounts of neuraminidase was prepared by affinity chromatography of Tween-ether disrupted FPV [9].

Fowl plague virus neuraminidase was isolated and purified as described [10].

For the preparation of *indicator virus* influenza virus A/PR8 in 0.01 M phosphate buffer solution, pH 7.2, was incubated for 30 min at 56 °C. Neuraminidase activity was destroyed.

Bromelain treated fowl plague virus was prepared according to Bachmayer and Schmidt [11]. Purified virus was incubated at 4 °C for 24 hours with bromelain and the virus particles separated by sucrose density gradient centrifugation. This virus preparation contained hemagglutinating but no measurable amounts of neuraminidase activity.

Vibrio cholerae neuraminidase (RDE) was purchased from Behringwerke AG, Marburg.

Glycoproteins. Fetuin was prepared according to Hamm and Puck [12]. Other serum glycoproteins, α_1 acid glycoprotein, α_2 macroglobulin, α_2 HS-glycoprotein, β_2 glycoprotein I [13] were obtained in purified form from Dr. Schwick, Behringwerke AG, Marburg.

Treatment of erythrocytes with soluble glycoproteins. 1 ml of washed and packed human erythrocytes (type 0) was treated with 50 units of RDE for 2 hours at 37 °C and washed three times with saline. Treated erythrocytes were fixed with 1% solution of glutaraldehyde in PBS, in an ice bath for 30 min. The fixed cells were washed three times

with 0.1 M glycine buffer, pH 7.3, and saline. To 1 ml of a 10% suspension of fixed erythrocytes, 1 ml glycoprotein solution (2 mg/ml) and 0.025 ml oleic acid-Na (10% w/v solution in saline)) were added. The reaction mixture was incubated at 40 °C for 30 min and the cell suspension washed with saline. Preliminary experiments showed that glycoproteins did not attach to the surface of red cells; whereas after complexing with oleic acid-Na, these substances were efficiently introduced into the cell surface. Addition of oleic acid-Na alone did not influence virus adsorption.

Adsorption and elution experiments. For adsorption 0.1 ml packed glycoprotein coated erythrocytes were mixed with 1 ml of virus preparations and kept for 30 min in an ice bath. The reaction mixture was centrifuged for 10 min at 3000 rpm and the supernatant assayed for hemagglutinating activity. The packed erythrocytes were washed three times with saline, resuspended in 1 ml NaCl solution, and incubated for 3 hours at 37 °C to elute the adsorbed virus. If spontaneous elution did not occur, 50 units of RDE were added to the incubation mixture.

Hemagglutination (HA)- and hemagglutination inhibition (HAI) tests. HA assays were performed in plastic plates according to standard procedures. For the HAI test, the method described by Davenport *et al.* [14] was used.

Virus plaque assay. Plaque tests were performed with embryonic chicken cells [15].

Determination of neuraminidase activity. Neuraminidase activity was measured with bovine sialyllactose as substrate as described before [16]. The amount of neuraminadase which liberated 1 μM sialic acid from the substrate/min at 37 °C was defined as one enzyme unit.

Results

Adsorption of FPV to fetuin-coated erythrocytes

In preliminary experiments we showed that erythrocytes fixed with glutaraldehyde adsorb myxoviruses nearly as efficiently as untreated red blood cells. After treatment of cells with RDE and subsequent fixation with 1% glutaraldehyde, the stabilized erythrocytes could not adsorb FPV. However, the binding capacity for the virus was restored, when treated erythrocytes were coated with fetuin. In controls virus particles were not adsorbed, if the fetuin-coated cells were treated with different neuraminidases, either RDE or FPV enzyme (Table I). At 37 °C the NANA of fetuin-coated cells was cleaved and the virus particles were eluted, and the erythrocytes were no more able to adsorb FPV (Table II).

Table I. Adsorption of fowl plague virus to native and fetuin-coated erythrocytes. 1 ml of FPV (128 HA units) were mixed with 0.1 ml packed erythrocytes, incubated for 30 min at 4 °C, the erythrocytes centrifuged and the supernatant tested for HA units.

Erythrocytes	after Adsorption HA Units
native	<2
neuraminidase-treatet and glutaraldehyde fixed	128
fetuin-coated	<2
native, neuraminidase-treated	128
fetuin-coated, neuraminidase-treated	128

Table II. Adsorption and elution of FPV to native and fetuin-coated erythrocytes. 128 HA units of FPV were adsorbed for 30 min at 4 °C and eluted for 120 min at 37 °C.

	HA Units	
	Native Erythrocytes	Fetuin Erythrocytes
after adsorption	<2	<2
after elution	96	128

The capacity of fetuin-coated erythrocytes to adsorb virus particles depends on the concentration of fetuin. 100 μg of fetuin per 0.1 ml packed erythrocytes were sufficient to adsorb 128 HA units of virus. The amount of virus bound to erythrocytes cannot be increased by using a higher concentration of fetuin.

Table III. Adsorption capacity of fetuin-coated erythrocytes. Erythrocytes were treated with varying amounts of fetuin. After adsorption for 30 min at 4 °C the non-adsorbed virus was determined in the HA test.

μg Fetuin/0.1 ml Packed Erythrocytes	FPV-HA Units after Adsorption
500	<2
200	<2
100	<2
50	4
20	8
10	16
5	32
2	64
1	110
0.5	128
0	128

In comparative studies with different enveloped viruses, only myxoviruses were found to be adsorbed to fetuin-coated cells. Influenza A/PR8, influenza A2/Asia, virus N, Italien" and "Beaudette" strains of NDV, Sendai virus, Sindbis virus, and VSV were used for the corresponding experiments. With the orthomyxoviruses about 128 HA units, and with both NDV strains only 40 HA units were adsorbed. After addition of Sindbis and VSV and centrifugation of fetuin-coated erythrocytes the same plaque counts were found in the supernatant as in control preparations.

Adsorption of FPV to erythrocytes coated with other glycoproteins

In the next series of experiments, we examined whether glycoproteins, which do not inhibit hemagglutination, would act as virus receptors. Purified serum glycoproteins were conjugated to the surface of erythrocytes.

The results presented in Table IV show that all glycoproteins tested were capable of adsorbing FPV, irrespective of their property to inhibit hemagglutination. Receptor function disappeared after treatment of the glycoprotein coated cells with neuraminidase.

Table IV. Adsorption of FPV to erythrocytes coated with different glycoproteins. Adsorption was done as described in Table I. The HA inhibition test was carried out with influenza A/PR8 indicator virus and the determination of NANA according to Drzeniek *et al.* [16].

Glycoprotein	NANA-content [%]	HA-inhibition	Adsorbed HA Units
α_1 acid glycoprotein (orosomucoprotein)	9.9	64	64
α_2 macroglobulin	0.85	2	32
α_2 HS glycoprotein	5.1	2	128
β_2 glycoprotein I	4.2	2	64
fetuin	7.5	256	128
note	—	—	2

Virus components responsible for adsorption

In order to identify whether the hemagglutinin component of the virus surface is adsorbed to receptors or whether binding of the virus is mainly an enzyme substrate interaction, adsorption of these 2 components to native and fetuin-coated erythrocytes was investigated.

No significant adsorption of soluble FPV neuraminidase to native or fetuin erythrocytes was observed at 4 °C. Within the limits of the test procedure adsorption of the enzyme could not be detected even if the reaction time was reduced to one minute. In Table V is presented the result of an experiment in which the amount of neuraminidase was approximately equal to that required for saturation of the receptors. But no neuraminidase was found to be adsorbed also when higher enzyme concentrations (up to 500 enzyme units) were employed.

Table V. Adsorption of FPV neuraminidase to native and fetuin-coated erythrocytes. FPV neuraminidase containing 8.6 enzyme units was mixed with 0.1 ml packed erythrocytes and incubated at 0 °C. After centrifugation of the erythrocytes the neuraminidase content in the supernatant was determined.

Adsorption Time	Enzyme Units after Adsorption	
	Native Erythrocytes	Fetuin Erythrocytes
1	7.0	8.1
5	7.1	7.1
10	7.3	7.1
15	7.9	7.5
30	7.5	7.6

On the other hand, purified preparations of all neuraminidase-free hemagglutinins were adsorbed to native and fetuin-erythrocytes. In these experiments we used influenza virus A/PR8 heated for 30 min at 56 °C (indicator virus), bromelain-treated FPV, and neuraminidase-free soluble FPV hemagglutinin. Hemagglutinins of several preparations could not be eluted from the cells by incubating the reaction mixture at 37 °C for 3 hours. However, the elution was achieved by RDE or FPV neuraminidase treatment. The corresponding results

Table VI. Adsorption of neuraminidase-free hemagglutinins to or from native and fetuin-coated erythrocytes, respectively.

Erythrocytes	Treatment	Bromelain treated	FPV Sol. Hemaggl.	Indicator Virus
	control	256	128	256
native	after adsorption	2	2	2
	after elution without neuram.	2	2	2
	after elution with neuram.	128	128	128
fetuin-coated	after adsorption	2	2	2
	after elution without neuram.	2	2	2
	after elution with neuram.	128	128	128

are presented in Table VI. The adsorption was inhibited when the native or fetuin erythrocytes were pretreated with RDE.

The native erythrocytes loaded with the neuraminidase-free hemagglutinin were agglutinated by specific anti-hemagglutinin sera; this means that the hemagglutinin was firmly attached to the surface of erythrocytes.

Discussion

The results obtained are in accordance with the assumption that NANA is the active principle of the myxovirus receptor on the surface of erythrocytes. Since it was possible to exchange the receptor of erythrocytes by other NANA-containing glycoproteins, it is evident that the entire structure of the glycoproteins is not essential for attachment of virus particles. The finding that glycoproteins, which do not inhibit hemagglutination, can fully function as receptors, supports Gottschalk's suggestion [1] that only a tight fit between the complementary structure of the virus surface and the inhibitor blocks hemagglutination. Attachment of virus particles to the surface of cells, coated with non-inhibitory glycoproteins, does not require this high degree of complementarity necessary for a competition of hemagglutination inhibition. It may be noted, however, that the various glycoproteins do not adsorb all virus strains to the same extent, which probably reflects the number of NANA molecules exposed on these substances and therefore at the surface of the red cells.

For virus adsorption an enzyme-substrate interaction of virus and receptor is apparently not important. In accordance with previous findings [16], significant adsorption of pure neuraminidase was not observed. On the other hand, neuraminidase-free hemagglutinins, *e. g.* indicator virus, bromelain-treated virus and soluble hemagglutinin, are completely adsorbed to native or fetuin-treated erythrocytes. This is in accordance with previous findings of Schulze [17], who found that neuraminidase-free influenza virus particles retained their infectivity. By increasing the incubation temperature a spontaneous elution of neuraminidase-free hemagglutinins did not occur. Hemagglutinin was eluted only when NANA, which binds the hemagglutinin, was cleaved by neuraminidase, which was added to the hemagglutinin erythrocyte complex. These results agree with findings that influenza virus adsorption was not impaired by antineuraminidase serum [2, 3] and the elution was inhibited in the presence of the serum [10].

It is noteworthy that the NANA-containing substrate for neuraminidase is also the active site for the binding of the hemagglutinin. Therefore hemagglutinin, but not the neuraminidase, seems to be solely responsible for the virus attachment to the cell surface. The nature of the binding of hemagglutinin to NANA is electrostatic attraction as will be described in a separate communication.

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