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The influence of dextran sulfate on influenza A virus fusion with erythrocyte membranes

Andreas Herrmann[a], Thomas Korte[a], Klaus Arnold[b] and Bärbel Hillebrecht[a]

[a]*Institut für Biophysik, Fachbereich Biologie, Humboldt-Universität Berlin, Berlin, Germany and*
[b]*Institut für Biophysik, Fachbereich Medizin der Universität Leipzig, Leipzig, Germany*

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Summary

Dextran sulfate suppresses the low pH-induced fusion of influenza virus A/Brazil 11/78 with erythrocyte membranes, as shown by fluorescence dequenching assay, using the fluorophore octadecylrhodamine B chloride (R18). Inhibition of fusion was maximal at pH 5.0, while at higher pH values (>5.6) fusion was not affected. Hemolysis of intact red blood cells by influenza A virus at low pH values is also prevented by dextran sulfate. The inhibiting effect of the polymer is mainly ascribed to repression of virus attachment. Evidence is given that the conformational change of the virus envelope protein hemagglutinin (HA) responsible for triggering fusion is not affected by the polymer.

Influenza; Erythrocyte membrane; Fusion; Dextran sulfate

Introduction

Sulfated polysaccharides, such as dextran sulfate, have recently been shown to be highly effective inhibitors of HIV-1 and HIV-2 replication in vitro (Ito et al., 1987; Ueno and Kuno, 1987; Mitsuya et al., 1988; Baba et al., 1988a,b; Biesert et al., 1988, Tochikura et al., 1989). In addition, sulfated polysaccharides proved to have a broad spectrum of antiviral activity against enveloped

Correspondence to: A. Herrmann, Institut für Biophysik, Fachbereich Biologie, Humboldt-Universität Berlin, Invalidenstr. 42, O-1040 Berlin, Germany. Fax: 491-30-2897241.
Abbreviations: HIV, human immunodeficiency virus; RBC, red blood cell(s); R18, octadecylrhodamine B chloride; FDQ, fluorescence dequenching.

viruses as, e.g., herpes simplex virus, human cytomegalovirus, vesicular stomatitis virus (Baba et al., 1988c). Since dextran sulfates were inhibitory to HIV and other enveloped viruses at concentrations below their anti-coagulant threshold, they are regarded as potential drugs for the chemotherapy of virus infection due to their low toxicity (Baba et al., 1988c, 1990b).

Much attention has been given to the investigation of the mechanism of the antiviral activity of dextran sulfate and other related polysaccharides. The mode of inhibition of virus replication by dextran sulfate was attributed to an inhibition of virus adsorption to the cell membrane, the first step in virus infection (Mitsuya et al., 1988; Baba et al., 1988a; Schols et al., 1989; Ohki et al., 1991). For instance, it was suggested that dextran sulfate may block interaction between viral surface glycoprotein gp120 and the CD4 receptor (Yoshida et al., 1988). Dextran sulfate did not interfere with the binding of monoclonal antibodies to the CD4 receptor (Baba et al., 1988a; Bagasra and Lischner, 1988; Thiele et al., 1989). Therefore, it was proposed that dextran sulfate has a direct effect on virus adsorption, which may be attributed to a affinity of dextran sulfate for gp120 or gp41 of the HIV envelope (Bagasra and Lischner, 1988).

To elucidate the mode of action of dextran sulfate on enveloped virus fusion we decided to investigate the influence of dextran sulfate on influenza virus fusion with natural membranes, since influenza A virus fusion is by far the best characterized of enveloped virus fusion. Influenza A virus infects host cells by attachment of its envelope to cell surface receptors, followed by endocytosis and fusion of the viral membrane with the endosomal membrane at acidic pH levels, delivering its nucleocapsid (White et al., 1983). Both the initial binding and the fusion are mediated by the influenza virus spike protein, hemagglutinin (HA) (Gething and Sambrock, 1981). The fusion is mediated by the hemagglutinin following a pH-dependent conformational change of this protein (Skehel et al., 1982; Doms et al., 1985). It has proven convenient to study fusion between influenza virus and biological membranes by binding virus to the readily available erythrocyte membrane and regulating the fusion reaction by control of the buffer pH (Clague et al., 1992; Stegmann et al., 1986).

Recently, it has been reported that dextran sulfate inhibits fusion of influenza A virus with unilamellar liposomes (Lüscher-Mattli and Glück, 1990). A similar observation has been made for fusion of Sendai virus with liposomes (Arnold et al., 1990) and erythrocyte membranes (Ohki et al., 1991). However, it remains to be established whether the effect of dextran sulfate on fusion might be ascribed to an inhibition of a conformational change of viral fusion protein responsible for initiation of fusion (Lüscher-Mattli and Glück, 1990) and/or to a suppression of virus attachment.

In this study we examined the influence of dextran sulfate on influenza A virus fusion with erythrocyte membranes using a fluorescence dequenching assay, based on the relief of self-quenching of the 'lipid-like' probe octadecylrhodamine (R18) incorporated into virus membranes (Keller et al.,

1977; Hoekstra et al., 1984; Pritzen and Herrmann, 1988). This assay directly monitors fusion. Binding of influenza virus is mediated by neuraminic acid residues of the glycocalyx of erythrocytes, which are typical receptors for influenza virus. Low pH-induced fusion was monitored at 37°C by acidification of the suspension medium.

Experimental procedures

Materials

Octadecylrhodamine B chloride (R18), 4-heptadecyl-7-hydroxycoumarin and bis-ANS were purchased from Molecular Probes (Junction City, OR). Dextran sulfate (M_r 8000) was from Sigma. Fresh blood from healthy donors was obtained from the Blood Bank, Berlin-Lichtenberg, and was used within 3 days after sampling (ACD storage medium). Purified influenza virus A/Brazil 11/78 was kindly provided by Dr. A. Lesnau from Zentralinstitut für Hygiene, Mikrobiologie und Epidemiologie (Berlin).

Red blood cell and ghost preparation

After removal of buffy coat and plasma red blood cells (RBC) were washed three times in phosphate-buffered saline (PBS, pH 7.4). Unsealed erythrocytes were prepared according to Dodge et al. (1963).

Labeling of virus

2.5 μl of a 2 mM stock solution of R18 in ethanol was added with rapid vortexing to 0.5 ml of A/Brazil 11/78 (1 mg virus protein/ml). After incubation for 30 min at room temperature (in the dark) virus was washed with ice-cold PBS to remove unbound R18, and resuspended to a concentration of 1 mg/ml. Protein concentration of ghosts as well as of viruses was determined according to Lowry.

Virus binding to ghost membranes

150 mM NaCl solution buffered with 5.8 mM sodium phosphate (pH 7.4) or 20 mM sodium acetate (pH 4.8–6.0) was used. To 170 μl buffered saline (0°C) of a given pH (pH 4.8–7.4) 10 μl of R18-labeled A/Brazil 11/78 and 20 μl ghosts (protein concentration of 6 mg/ml) were added and incubated for 5 min on ice with gently vortexing. Usually, virus was preincubated with dextran sulfate for 5 min prior to addition of ghosts. In some experiments ghosts were preincubated with dextran sulfate or dextran sulfate was added after binding of virus to ghost membranes.

Fusion analysis

Fluorescence dequenching of R18-labeled virus attached to RBC was measured using a double-beam spectrofluorometer Perkin-Elmer 512, equipped with a chart recorder and a thermostated sample chamber. Fusion was

triggered by transferring 200 μl of ice-cold virus-ghost suspension to a quartz cuvette containing 1.8 ml of prewarmed (37°C) buffered saline of the same pH (pH 4.8–7.4) and dextran sulfate concentration. The suspension was stirred continuously with a 2 × 8 mm Teflon-coated magnetic stir bar. Fusion was monitored continuously by measuring fluorescence dequenching for 10 min (λ_{ex} = 560 nm, λ_{em} = 590 nm, cut-off filter 570 nm), after which Triton X-100 (0.5% final concentration) was added to obtain maximum R18 fluorescence F_{max}. The percentage of fluorescence dequenching, FDQ, was calculated as described previously (Blumenthal et al., 1987):

$$\% \text{ FDQ} = 100 \times [F(t) - F(0)] / [F_{max} - F(0)] \quad (1)$$

where F(0) and $F(t)$ correspond to the fluorescence intensity of the virus before starting fusion and the fluorescence intensity at a given time t, respectively. Values of FDQ presented in Results refer to the FDQ at 10 min (see Results). Control samples, without addition of dextran sulfate, were measured in each set of experiments.

Binding assay

The influence of dextran sulfate on the amount of influenza virus bound to erythrocyte membranes was measured at 0°C at various pH values. In order to allow fast and clear separation of erythrocytes and virus by low-speed centrifugation, intact red blood cells were used instead of erythrocyte ghosts*. To 1.7 ml of ice-cold buffered saline of a given pH and dextran sulfate concentration 20 μl of R18-labeled influenza virus was added. After 5 min of incubation at 0°C by gently vortexing, 0.5 ml of the virus suspension was taken and the fluorescence intensity obtained in the presence of 0.5% Triton X-100 was used as a measure of the total amount present. To the remaining 1.2 ml of virus suspension 40 μl of red blood cells (10^9 cells/ml) were added and incubated for 5 min with gentle vortexing. After low-speed centrifugation (max 1000 × g, 2 min total) 0.5 ml of the supernatant were taken to measure the amount of unbound virus (fluorescence intensity after addition of 0.5% Triton X-100).

Surface potential of influenza virus A/Brazil 11/78

The surface potential of influenza virus was measured according to the procedure of Fromherz (1989) using the membrane-inserted fluorophore 4-heptadecyl-7-hydroxycoumarin whose fluorescent properties are sensitive to pH. The method is based on the change of the interfacial proton concentration, leading to a corresponding shift of the apparent pK (pK_{app}) of the fluorophore. The pK_{app} is derived from the dissociation degree of the probe, which is

*We have concluded from electrophoretic measurement of human erythrocytes that the number of sialic acid groups, as well as their arrangement perpendicular to the membrane surface, is not affected by ghost preparation (Herrmann et al., 1986).

obtained from the fluorescence intensity of the basic form at 450 nm (λ_{ex} = 378 nm) normalized to the intensity at high pH values of the suspension medium (for details: see Fromherz, 1989). The surface potential, Φ_0, is obtained by equation (2):

$$\Phi_0 = -2.3\,kT\cdot(1/e_0)\,[pK_{app} - pK^{\circ}_{app}] \quad (2)$$

where kT, e_0 and pK°_{app} correspond to the thermal energy, the proton charge and the apparent pK in a membrane of vanishing potential, respectively (pK°_{app} of 8.65 for egg-PC vesicles) (Pal et al., 1983). Influenza virus was labeled with the Coumarin fluorophore at a dye/lipid ratio of 1:200 by incubation in the dark at room temperature for 30 min.

Binding of bis-ANS to influenza virus

Influenza virus A/Brazil 11/78 (0.5 mg/ml) was preincubated in the presence or absence (control) of dextran sulfate (2.5 nM to 2.5 μM) at pH 5.0 and pH 7.4, respectively (0°C). After 5 min incubation, 40 μl of the virus suspension were added to 2 ml of pre-warmed buffer (37°C, pH 5.0 or pH 7.4) with the same concentration of dextran sulfate containing 2.85 μM of bis-ANS. Binding of bis-ANS to influenza virus was followed by measuring the fluorescence intensity at λ_{em} = 400 nm (excitation λ_{ex} = 490 nm). The suspension was stirred continuously with a 2 × 8 mm Teflon-coated magnetic stir bar. Dextran sulfate did not affect the fluorescent properties of bis-ANS dissolved in aqueous media.

Hemolysis assay

After pretreatment of unlabeled A/Brazil 11/78 with dextran sulfate (0°C, pH 4.8–6.0) for 5 min, 20 μg of virus were bound to 10^8 intact red blood cells in 200 μl of buffered saline (pH 4.6–6.0, 0°C) in the presence of dextran sulfate. After 5 min incubation 1.8 ml of pre-warmed buffer (37°C, pH 4.8–6.0) containing dextran sulfate were added. Following an incubation at 37°C for 5 min the suspension was centrifuged and the supernatant (0.2 ml) was diluted in 1 ml of 0.5% NH_4OH and the extinction was measured at 540 nm. The reference (100% hemolysis) was determined by mixing of 0.2 ml of the suspension with 1 ml of 0.5% NH_4OH.

Susceptibility of HA to protease in the intact virus

Protease susceptibility of HA was tested according to Puri et al. (1990). After incubation of virus (50 μg in 0.5 ml) at pH 7.4 or pH 5.0 for 15 min, 37°C, in the absence or presence of dextran sulfate (2.5 μM), virus was treated with 30 μg of proteinase K (Sigma) at 37°C for 30 min at pH 7.4. Protein was precipitated by addition of an equal volume of 20% trichloroacetic acid and sedimented by spinning at 20 000 × g for 5 min (Biofuge Heraeus 28RS). The pellets were washed, diluted (1:4) and boiled for 4 min in a buffer containing 2% sodium dodecyl sulfate, 20% mercaptoethanol, 10% glycerol, and 62.5

mM Tris (pH 6.8). The samples were run on SDS-12% polyacrylamide gels under reducing conditions according to Laemmli.

Results

Influence of dextran sulfate on fluorescence dequenching of influenza virus

After triggering fusion of A/Brazil 11/78 with erythrocyte membranes by low pH at 37°C a rapid fluorescence increase was observed, which almost reached its final value after 10 min (Fig. 1, control; kinetics not shown, see Pritzen et al., 1990). The typical pH-dependence of fluorescence dequenching for fusion of influenza virus with appropriate targets (such as erythrocyte membranes) was observed for A/Brazil 11/78 (Fig. 1, control). FDQ was maximal at pH 5.0, 37°C in agreement with previous results (Pritzen et al., 1990). Although virus was bound at pH 7.4 no dequenching was observed at 37°C, suggesting that the fluorescence dequenching is caused by fusion and not by non-specific R18 transfer to erythrocyte membranes from attached, but non-fused, viruses. Therefore, FDQ reflects fusion of influenza.

Pretreatment of A/Brazil 11/78 with dextran sulfate (M_r 8000) at pH 5.0, 0°C before binding to ghosts reduces FDQ at concentrations as low as 0.1 μM dextran sulfate. Typical experiments are shown in Fig. 2 (circles).

The same repressing effect of dextran sulfate on influenza fusion was

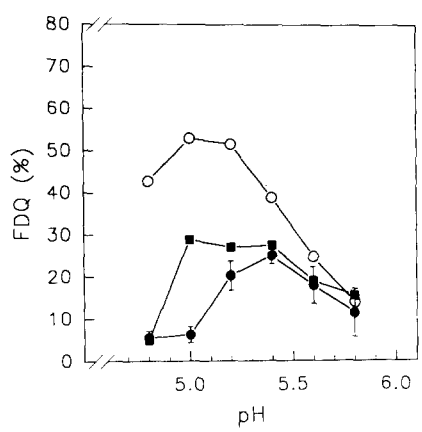

Fig. 1. The pH-dependence of fusion of R18-labeled A/Brazil 11/78 with erythrocyte membranes at 37°C. Virus was pretreated with dextran sulfate at a given pH, 0°C, for 5 min before binding to ghost membranes (○, control; ■, 0.3 μM; and ●, 2.5 μM dextran sulfate, M_r 8000). Fusion was initiated by transferring 200 μl of the virus-ghost suspension to a quartz cuvette containing 1.8 ml of pre-warmed (37°C) buffer solution of the same pH and concentration of dextran sulfate (see Experimental procedures). Values of FDQ were calculated according to Eqn. 1 and refer to those measured 10 min after triggering fusion. For clearness the standard error of estimate (three independent experiments) is given only for the sample of 2.5 μM dextran sulfate.

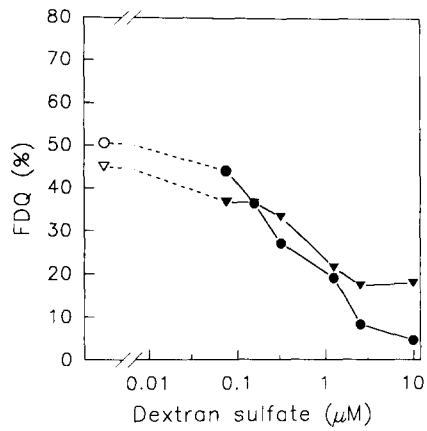

Fig. 2. The influence of dextran sulfate on the fusion of influenza virus A/Brazil 11/78 with erythrocyte membranes at pH 5.0, 37°C. Virus (●) or ghosts (▼), respectively, were pretreated with dextran sulfate at pH 5.0, 0°C, for 5 min before binding of virus to ghost membranes. (○, ▽), control, absence of dextran sulfate.

observed by preincubation of ghost membranes with dextran sulfate before binding of virus (Fig. 2, triangles); only at a higher concentration (>3 μM) preincubation of virus was more effective in suppressing fusion. However, the inhibition of virus fusion was less pronounced – but still significant – when

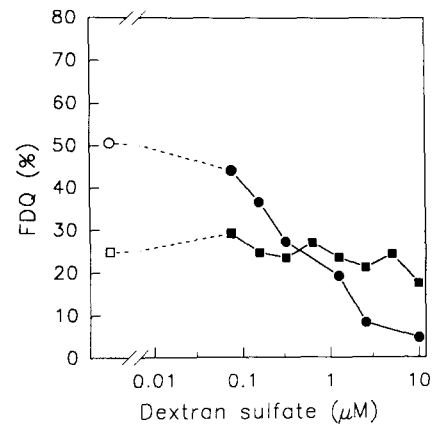

Fig. 3. The influence of dextran sulfate on the fusion of influenza virus A/Brazil 11/78 with erythrocyte membranes at pH 5.0 (○, ●) and 5.8 (□, ■), 37°C. Virus was pretreated with dextran sulfate at the desired pH, 0°C, for 5 min before binding of virus to ghost membranes. Fluorescence dequenching was initiated and measured, as described in legend of Fig. 1. (○, □), control, absence of dextran sulfate.

dextran sulfate was added 5 min after virus binding to the target (data not shown).

The suppressing effect of dextran sulfate on influenza fusion was established in the pH range 4.8 to about pH 5.5 (Fig. 1). The maximum of FDQ was shifted to a higher pH value by increasing the concentration of dextran sulfate. At higher, suboptimal pH values (>5.6) fusion was not reduced by dextran sulfate, even at rather high concentrations of the polymer (Figs. 1 and 3). Similar results were obtained when preincubation with dextran sulfate and binding to erythrocyte membranes were performed at pH 7.4.

An inhibiting effect on fusion similar to that of dextran sulfate was not observed when using dextran (M_r 9400) or poly(ethylene glycol) (M_r 8000). At concentrations up to 10 μM these uncharged polymers did not affect fusion (data not shown). However, at concentrations in the order of 5 to 10 wt% these polymers accelerated and enhanced low pH induced fusion of influenza virus with red blood cells (Herrmann, Clague, Blumenthal, unpublished results) which was not found for dextran sulfate.

Influence of dextran sulfate on virus binding to red blood cells

Attachment of A/Brazil 11/78 to intact red blood cells in the presence of dextran sulfate was dependent on pH (Fig. 4). Suppressing of virus binding by dextran sulfate was observed between pH 4.8 and 5.4, maximal at pH 5.0**. The

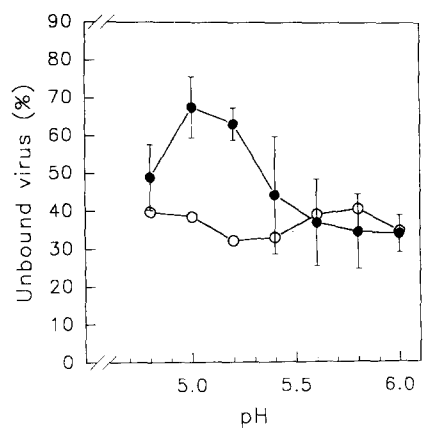

Fig. 4. pH dependence of virus binding to membranes of intact red blood cells in the absence (○) and presence of dextran sulfate (●, 2.5 μM), respectively. Virus was pretreated with dextran sulfate at the desired pH, 0°C, for 5 min before binding to erythrocyte membranes. Binding was measured as described in Experimental procedures. For clearness the standard error of estimate (three independent experiments) is given only for the sample of 2.5 μM dextran sulfate.

**Using the two-tailed t-test for unpaired observations, virus binding was significantly different (confidence level 0.10) at pH 5.0 ($P=0.076$) and pH 5.2 ($P=0.0149$).

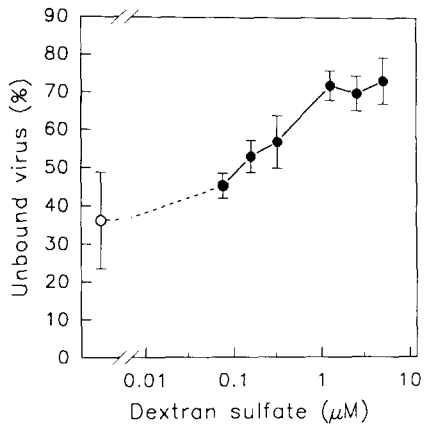

Fig. 5. The influence of dextran sulfate concentration on binding of influenza virus A/Brazil 11/78 to membranes of intact red blood cells at pH 5.0, 0°C. Virus was pretreated with dextran sulfate at pH 5.0, 0°C, for 5 min before binding to erythrocyte membranes. Binding was measured as described in Experimental procedures. The standard error of estimate (three independent experiments) is given. (○), control, absence of dextran sulfate.

shape of the pH-dependence of binding in the presence of the polymer (Fig. 4) is similar to the pH-dependence of FDQ of the control (Fig. 1). Above pH 5.4 no significant difference in binding between control and samples containing dextran sulfate was observed. In the absence of the polymer no pH dependence of virus attachment was found. Values obtained at pH 7.4 (data not shown) did not differ from those between pH 4.8 to 6.0 in accordance with earlier observations (Maeda and Ohnishi, 1980; Yoshimura et al., 1982; Loyter et al., 1988).

At pH 5.0 a continuous decrease of bound virus was found by increasing dextran sulfate concentration (Fig. 5) paralleling the decline of FDQ (Fig. 2).

Influence of dextran sulfate on the surface potential of influenza virus

We have determined the membrane surface potential of influenza virus A/Brazil 11/78 with the fluorophore 4-heptadecyl-7-hydroxycoumarin (Fromherz, 1989) which shows pH-dependent spectral changes. From NMR measurement it has been concluded that the probe is located in the plane of the phosphates of lipid head groups (Fromherz, 1989). It has been used successfully to measure the surface potential of vesicular stomatitis virus (Pal et al., 1983). The fluorophore was found to be incorporated quite easily into the membranes of A/Brazil 11/78. In order to gain maximal sensitivity to changes of the surface potential we have measured the fluorescent spectrum at a pH close to the pK measured for egg-PC (pK°_{app} = 8.65; Pal et al., 1983) (see Table 1). Dextran sulfate has only a minor influence on the negative surface potential of A/Brazil 11/78. Even at rather high concentrations of dextran sulfate (10 μM and 20

TABLE 1

Influence of dextran sulfate on the surface potential of influenza virus A/Brazil 11/78

Dextran sulfate concentration (μM)	pK_{app}	Surface potential Φ_0 (mV)
0 (control)	8.86	−13.4
10	8.89	−14.9
20	8.91	−15.9

The pK_{app} of the membrane-inserted fluorophore 4-heptadecyl-7-hydroxycoumarin was derived from its fluorescence spectrum measured at a pH of 8.35, 37°C. Φ_0 was calculated according to Eqn. 2 (for details see Experimental procedures).

μM) only a slight decrease of the surface potential was found. Although absolute values of the apparent pK-values from independent experiments differed (e.g., pK_{app} of the control (average ± S.D.) 8.85 ± 0.11 (n=4)) the same conclusions could be drawn in each case. Unfortunately, the probe does not allow the determination of the surface potential at low pH values (5.0).

Conformational change of HA

(a) *Protease digestion of HA* It has been shown that the conformational change of the HA can be assessed by protease digestion. Whereas the neutral form of HA is resistant to a variety of proteases, the acid conformation is susceptible to protease digestion (Doms et al., 1985; Puri et al., 1990). We examined the effect of dextran sulfate on the conformational change by proteinase K digestion, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis of intact virus before and after treatment with the enzyme. The HA_1 was clearly visible after exposure to the protease when the virus was not incubated at low pH. However, upon low-pH value treatment digestion of the virus with proteinase K led to the disappearance of HA_1. HA_1 vanished also when the low-pH value incubation of the virus was performed in the presence of 2.5 μM dextran sulfate, indicating that the conformational change was not affected by the polymer.

(b) *Binding of bis-ANS to virus membranes* It has been shown that bis-ANS is strongly adsorbed to hydrophobic binding sites in proteins (Rosen and Weber, 1969) and to several lipids, but with a lower affinity (Lambers et al., 1984). The quantum yield of this fluorescence probe depends on the polarity of the environment. It is virtually non-fluorescent in aqueous solutions, but becomes strongly fluorescent in apolar solvents or when it is bound to hydrophobic sites in proteins (Rosen and Weber, 1969).

Binding of bis-ANS to A/Brazil 11/78 showed a strong pH dependence at 37°C, which was similar to the pH dependence of FDQ (Fig. 1, control). Between pH 7.4 and 5.8 fluorescence intensity of bound bis-ANS was almost constant. However, at pH values <5.8 fluorescence intensity increased, with a maximum at pH 5.0 (Fig. 6).

We suggest that this fluorescence increase reflects binding of bis-ANS to

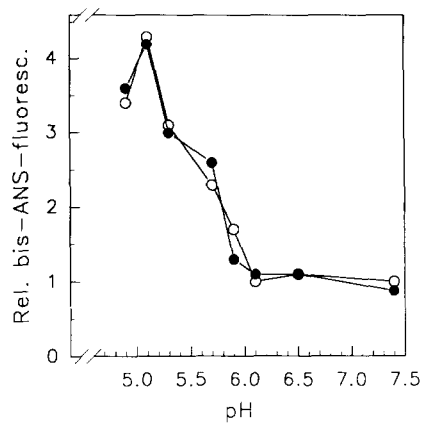

Fig. 6. The influence of dextran sulfate on the fluorescence intensity of bis-ANS in the presence of A/Brazil 11/78; ○, control, no dextran sulfate; ●, 2.5 μM dextran sulfate. Fluorescence was normalized to the value of the control (no dextran sulfate) at pH 7.4. bis-ANS fluorescence was measured as described in Experimental procedures.

hydrophobic binding sites exposed by a conformational change of HA of influenza virus. This conclusion is based on the following observations: (i) HA of A/Brazil 11/78 undergoes an irreversible conformational change at low pH values, as shown by protease digestion (see above). Similarly, the enhanced binding of bis-ANS to influenza virus at low pH was not reversible after adjusting the pH to 7.4 (data not shown). This is a strong indication that the fluorescence increase at low pH values is related to the conformational change of the HA. (ii) This suggests also that the pH-dependent enhancement of binding and fluorescence of bis-ANS in the presence of virus cannot be explained solely by protonation of negatively charged protein residues. (iii) Binding of bis-ANS to influenza virus was pH-independent (pH 4.8–7.4) after cleavage of the external part of HA with bromelain (Korte and Herrmann, unpublished results).

We have compared the fluorescence behavior of bis-ANS bound to influenza virus in the absence (control) and presence of dextran sulfate (0.0025 μM to 2.5 μM) between pH 7.4 and 5.0 at 37°C. In any case, no significant influence of the polymer on the fluorescence of bis-ANS bound to the virus membrane could be established (Fig. 6).

Hemolysis

Fusion of influenza virus with red blood cells is often accompanied by the release of hemoglobin from the erythrocytes (hemolysis). We have examined the effect of dextran sulfate on the A/Brazil 11/78-induced hemolysis at low pH, 37°C. In Fig. 7 (pH 5.0) the results of a duplicate experiment show that the polymer significantly reduced the release of hemoglobin from intact erythrocytes. Although virus-induced hemolysis at low pH is more sensitive

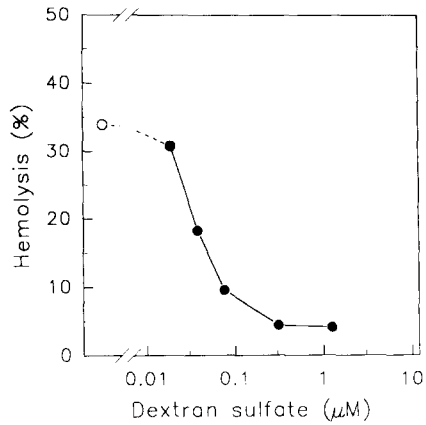

Fig. 7. Low pH induced hemolysis of RBC by A/Brazil 11/78 as a function of dextran sulfate concentration. Hemolysis was measured 5 min after lowering the pH to 5.0, 37°C, as described in Experimental procedures. (○), control, absence of dextran sulfate.

to dextran sulfate (compare also Fig. 1 and 8), the concentration range of dextran sulfate where a continuous decrease of hemolysis was observed is in the order of that observed for FDQ (Fig. 2).

The pH-dependence of the hemolytic activity of influenza virus (Fig. 8) closely follows its fusogenic activity. However, the maximum of hemolytic activity is slightly shifted to higher pH-values in comparison to the fusion activity. Dextran sulfate suppresses virus-induced hemolysis, in particular between pH 4.8 and 5.6, as shown in Fig. 8, for two different concentrations of the polymer (0.037 and 0.3 μM).

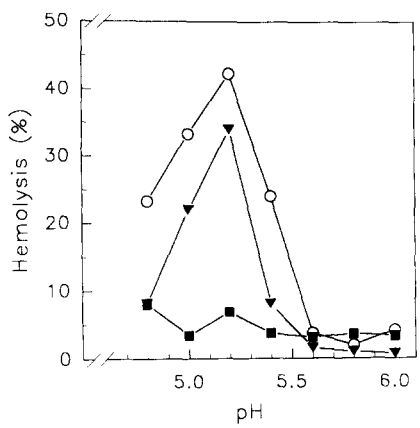

Fig. 8. pH-Dependence of A/Brazil 11/78-induced hemolysis of RBC at different concentrations of dextran sulfate; ○, control, no dextran sulfate; ▼, 0.037 μM and ■, 0.3 μM dextran sulfate. Hemolysis was measured 5 min after lowering to the indicated pH, 37°C, as described in Experimental procedures.

Discussion

The low pH induced fusion of influenza virus with erythrocyte membranes is suppressed by dextran sulfate. This is in accordance with previous observations on fusion of influenza virus with liposomes (Lüscher-Mattli and Glück, 1990) bearing no typical biological receptor for influenza virus attachment. We ascribe the inhibiting effect of the negatively charged polymer on virus fusion and virus-induced hemolysis of red blood cells mainly to an inhibition of virus attachment to the target membrane rather than to an interference with the conformational change in the HA as suggested by Lüscher-Mattli and Glück (1990).

Binding of dextran sulfate to membranes

It was suggested that dextran sulfate is bound via electrostatic interaction to influenza A virus surface proteins, hemagglutinin and neuraminidase, (Lüscher-Mattli and Glück, 1990) which both are positively charged at pH 7 and below (Skehel and Schild, 1971). Indeed, positively charged amino groups of proteins are potent binding sites for dextran sulfate (Krumbiegel et al., 1988). We observed, by using a pH-sensitive fluorophore, a small change of the surface potential of the virus membrane after addition of dextran sulfate, which might indicate that the polymer is bound to the virus membrane. The minor effect on the surface potential suggests that the polymer is not bound to the membrane (lipid) surface, but rather to the external part of the envelope proteins. Steric constraints might contribute to an exclusion of the polymer from the lipid head group surface. However, the relevance of the negative charged sulfate groups of the polymer for its inhibitory effect is sustained by our observation that uncharged polymers did not affect the virus-induced fusion process under similar conditions. The inhibitory effect of dextran sulfate on Sendai virus fusion with erythrocyte ghosts was also attributed to a preferential binding of the polymer to positively charged groups of the external part of envelope proteins (Ohki et al., 1991). In contrast, binding of the polymer to erythrocytes was only weak, as judged from their aggregation behavior (Ohki et al., 1991) and electrophoretic mobility in the presence of dextran sulfate (Krumbiegel, personal communication). This is presumably related to the negatively charged neuraminic acid residues of the glycocalyx of the erythrocyte membrane.

Inhibition of virus-induced fusion by dextran sulfate

The inhibitory effect of dextran sulfate on envelope virus fusion has been attributed to a suppression of virus attachment and/or to an inhibition of interaction between the viral surface proteins and the target membrane. In particular, for influenza A virus it has been suggested that the low-pH induced conformational change of the HA essential for fusion is suppressed by the polymer (Lüscher-Mattli and Glück, 1990). It is known that the conformational change of HA at low pH is accompanied by the exposure of the

hydrophobic N-terminus of the HA2 polypeptides, the so-called 'fusion sequence', and the dissociation of the HA_1 top domains of the trimer from each other becoming susceptible to proteinase K (Skehel et al., 1982; Doms et al., 1985; White and Wilson, 1987; Stegmann et al., 1991). Stegmann et al. (1991) have proposed that even at low temperature (0°C) a fast primary conformational change of HA leads to the exposure of the fusion sequence sufficient to induce fusion with erythrocyte membranes; the permanent alteration of the top domain of HA_1 occurs only at elevated temperatures.

In contrast to the findings of Lüscher-Mattli and Glück (1990), we did not observe that the polymer interferes with the conformational change of the HA because: (i) the susceptibility of HA of low-pH treated influenza virus to proteinase K is not affected by dextran sulfate; (ii) the strong increase of bis-ANS fluorescence which is presumably due to the exposure of hydrophobic binding sites upon the conformational change of the HA at low pH is not altered by the presence of dextran sulfate.

We associate the diminished extent of influenza virus fusion in the presence of dextran sulfate to an inhibition of virus attachment to the erythrocyte membrane. The concentration dependence of the polymer-induced inhibition of virus binding to the target at pH 5.0 (Fig. 5) parallels that of the decline of fluorescence dequenching (Fig. 2). The decrease of virus binding might be explained by an increase of hydration of the virus surface caused by the polymer. Hydration repulsion is a major barrier to be overcome in membrane aggregation and fusion. It is already known that a typical feature of polymers such as dextran is the pronounced ability to bind water (Arnold et al., 1988). Moreover, sulfate ions increase the hydration of proteins (Bull and Breeze, 1976).

A rather surprising result was that the inhibition of attachment, as well as fusion, of virus was dependent on pH. At pH values optimal for fusion (pH 5.0) the effect of the polymer was maximal. At suboptimal pH (> 5.6) no significant differences between control and dextran sulfate-treated samples were established. Recently, it was demonstrated that the suppressing effect of dextran sulfate on fusion of Sendai virus with erythrocyte membranes is continuously enhanced by lowering the pH from 7.4 to 4.5 (Ohki et al., 1991). This was explained by an increased binding of dextran sulfate caused by protonation of negatively-charged residues of Sendai virus surface by lowering the pH. However, the effect of dextran sulfate on influenza virus fusion cannot be attributed only to a raised binding of the polymer by acidification of the suspension medium. In that case, we would expect a continuous drop in the number of bound virus. The bell-shaped curve (Fig. 4, 2.5 μM dextran sulfate) rather suggests that the pH-dependent binding of virus in the presence of the polymer is related to the conformational change of the HA, because it is similar to the pH-dependence of the fusion activity of A/Brazil 11/78 (Fig. 1 control) which directly reflects the change in the structure of HA. It has been shown by proteinase K sensitivity that, although the low pH induced conformation of HA is irreversible, the final amount of this form is pH-dependent (Doms et al.,

1985). In particular, since our binding experiments were done on ice, one may attribute the decreased binding of virus in the presence of dextran sulfate to the exposure of the hydrophobic N-terminus. However, at present we are not able to give a satisfying explanation for the observed pH-dependent binding of influenza virus in the presence of dextran sulfate.

In conclusion, our results confirm that inhibition of enveloped virus fusion by dextran sulfate is not specific for HIV, but can also be observed for other enveloped viruses. Sulfated polysaccharides inhibit the adsorption/fusion of some retroviruses (i.e., HIV-1, HIV-2, SIV) and myxoviruses (RSV, influenza A) but not others (i.e., influenza B, measles, parainfluenza-3). This inhibitory effect seems to be related to the presence of the tripeptide segment Phe-Leu-Gly in the envelope glycoproteins of the sensitive viruses (Hosoya et al., 1991). As shown in the present paper, dextran sulfate does not alter the conformational change of the influenza A, which is necessary for fusion.

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