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Dextran sulfate inhibits fusion of influenza virus and cells expressing influenza hemagglutinin with red blood cells

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The influence of dextran sulfate with molecular weights of 500000 and 8000 on binding and fusion of influenza virus (X31 strain) and of cells expressing influenza hemagglutinin (GP4F) with red blood cells (RBC) was investigated by spectrofluorimetry using virus and RBC labeled with the fluorescent dye octadecyl rhodamine B (R_{18}). There was no significant inhibition of binding of virus and GP4F cells to red blood cells by dextran sulfate, but the polymer strongly inhibited the low pH induced fusion. Virus-RBC fusion was completely blocked by the high molecular weight dextran sulfate at concentrations as low as 0.5 mg/ml. Inhibition of RBC-GP4F cell fusion by dextran sulfate in the same concentration range was not as pronounced but the effect was potentiated by Ca^{2+}. The polymer was only inhibitory when added at early steps of the fusion reaction, but the pH-induced conformational change of the hemagglutinin was not affected by dextran sulfate as measured by its susceptibility to proteolytic digestion. Removal of dextran sulfate after low pH-requiring steps allowed the system to fuse at neutral pH indicating that the inhibitory effect requires the continuous presence of dextran sulfate during the fusion reaction.

Introduction

Sulfated polysaccharides are known to inhibit the infectivity of several enveloped viruses [1,2]. In the past years special interest was focused on these substances because of their anti-HIV-1 effects and their potential chemotherapeutical use against AIDS [3,4]. The common explanation is that these macromolecules block the binding of virus to the target cells [5,6], but other factors involved in the entry of virus into cells might also play a role. In some recent publications fluorescence fusion assays have been used to study the influence of dextran sulfate on viral fusion [7,8]. The detailed understanding of the molecular mechanisms underlying the modulatory effect of sulfated polysaccharides on enveloped virus activity may be important for the development of new antiviral chemotherapeutic strategies and substances.

We used dextran sulfate of different molecular weights (8000 and 500000) as one representative of this class of macromolecules to investigate the influence on the binding and fusion of influenza virus (X31 strain) and influenza HA-expressing cells [9] with red blood cells. GP4F cells are a line of transformed NIH 3T3 cells that constitutively express uncleaved influenza hemagglutinin (HA0) [9]. By mild trypsinization this precursor is changed into the fusion competent viral spike protein. Influenza virus and GP4F cells can be prebound to RBC at neutral pH. The fusion of these complexes can then be induced by lowering the pH of the medium [10,11]. To monitor the fusion reaction we used the well-established R_{18}-fusion assay that is based on the self-quenching properties of this lipophilic dye [12]. Proteolytic digestion by proteinase K of dextran sulfate-treated virus was performed to examine the possible interference of dextran sulfate with the low pH induced conformational change of HA that is essential for the fusion reaction. We found that while dextran sulfate strongly inhibited fusion it did not significantly affect binding and had no effect on the conformational change of HA at low pH.

Materials and Methods

Materials. Octadecylrhodamine B chloride (R_{18}) was purchased from Molecular Probes (Junction City, OR), dextran sulfate with mean molecular weights of 8000

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Abbreviations: HA, hemagglutinin; R_{18}, octadecylrhodamine; DMEM, Dulbecco's modified Eagle's medium; DS8, dextran sulfate (MW 8000); DS500, dextran sulfate (MW 500000); RBC, red blood cell.

and 500000 were from Sigma (St. Louis, MO). Fresh blood from healthy donors was obtained from the NIH Blood Bank.

Virus. Influenza, X31 strain was obtained as described in Ref. 13. The virus was grown for 48 h at 37°C in the allantoic cavity of 10-day-old embryonated hen eggs. The allantoic fluid was harvested and centrifuged at $1000 \times g$ to remove cell debris, and then the virus was pelleted by centrifugation of the allantoic fluid for 50 min at $85000 \times g$. The pellet was dispersed in PBS and homogenized.

Cells. GP4F cells were grown at 37°C, 7% CO_2 to 80–90% confluency in DMEM supplemented with 10% fetal calf serum in 75-cm^2 plastic flasks. For cleavage of HA0 the cells were washed two times with DMEM (without fetal calf serum) and then incubated with 5 ml of 5 μg/ml trypsin and 0.22 mg/ml neuraminidase for 10 min at room temperature while still bound to the flask [11]. The reaction was terminated by addition of 10 ml DMEM with 10% fetal calf serum. After washing the cells the binding of RBC was performed (described below).

For preparing erythrocytes whole blood was centrifuged and after removal of plasma the red blood cells were washed three times with PBS (pH 7.4).

Labeling of influenza virus and prebinding to RBC. 1.25 μl of 1 mg/ml R_{18} (in ethanol) was added under vortexing to 0.5 ml virus suspension containing 0.3 mg protein. After incubation for 10 min at room temperature unbound R_{18} was removed by elution from a Sephadex G-25 PD10 column (Pharmacia, Piscataway, NJ). The labeled virus (0.3 mg protein) was then incubated with 0.8 ml of washed RBC (10^9 cells/ml) for 20 min on ice. After two washes the pellet was resuspended in 0.3 ml PBS and kept on ice until used for fusion assays.

Labeling of RBC with R_{18} and prebinding to GP4F cells. As described in ref. 11, 30–50 μl of 1 mg/ml R_{18} (in ethanol) was injected under vortexing to 10^9 RBC in 1 ml PBS. 10 ml PBS was added and the sample was incubated 30 min at room temperature in the dark. To remove unbound R_{18} 35 ml DMEM with 10% fetal calf serum was added and the RBC were further incubated for 15 min in the dark. The RBC were then washed five times with 50 ml PBS. 5 ml of the labeled RBC (10^7 cells/ml) were added to the GP4F cells still bound to the flask for about 6 min at room temperature. Unbound RBC were removed by six times gently washing with DMEM. The RBC-GP4F cell complexes were then lifted from the flask by treating with 0.5 mg/ml trypsin, 0.2 mg/ml EDTA in PBS for 12 min at 37°C. The cells were washed two times by centrifugation with PBS and resuspended in 0.5 ml cold PBS and kept on ice until used for fusion assays.

R_{18} fusion assay. The R_{18} fluorescence was measured using a SLM-8000 spectrofluorimeter (SLM-Aminco, Urbana, IL) at 560 and 590 nm excitation and emission wavelength, respectively. 2 ml of PBS buffer at preadjusted pH and 37°C was placed in the fluorescence cuvette and stirred. Microliter amounts of virus-RBC or RBC-GP4F cell complexes were rapidly added and the fluorescence was monitored ($F(t)$). The maximal dequenching was obtained by adding 0.1% Triton X-100 (F_T). The dequenching was calculated from the following equations [14]: percent of fluorescence dequenching = $F(t)/F_T \times 100$ (for virus-RBC fusion) and dequenching = $\{(F(t) - F_0)/(F_T - F_0) \times 100$ (for RBC-GP4F cell fusion; F_0 is the initial fluorescence).

Binding assays. GP4F cells were grown and the HA0 was cleaved as described above. The attached GP4F cells were incubated with 5 ml washed RBC (10^7 cells/ml) in the absence and presence of dextran sul-

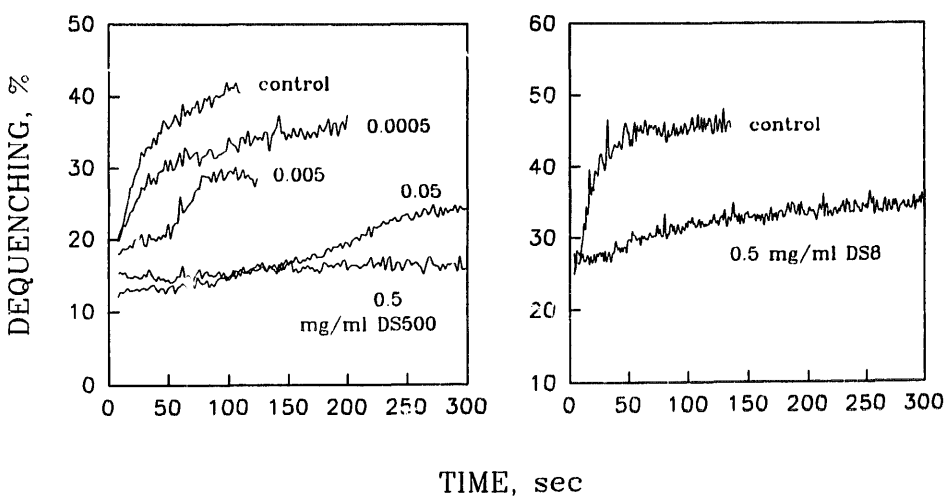

Fig. 1. Influence of DS500 (left) and DS8 (right) on the fusion between influenza virus and RBC. The fusion was initiated by adding the prebound virus-RBC complexes to PBS buffer at pH 5.0 and 37°C. The buffer contained different concentrations of dextran sulfate as indicated.

fate for 10 min at room temperature in PBS at pH 7.4. Then the complexes were gently washed three times with DMEM. The numbers of erythrocytes bound per GP4F cell were counted under a microscope.

To estimate the binding of virus to RBC 150 μl R_{18}-labeled influenza virus suspension containing 50 μg protein were mixed with 5 ml RBC ($2.5 \cdot 10^7$ cells/ml) in PBS (pH 7.4) in the absence and presence of dextran sulfate. After 20 min incubation at room temperature the RBC were spun down for 5 min at $1000 \times g$ and the supernatant (containing unbound virus) was aspirated. The pellet was washed by resuspending in cold PBS and spinning down again. The pellet was then resuspended in 1 ml PBS. 20 μl of this suspension were added to 2 ml PBS in the presence of 0.1% Triton X-100 to yield infinite dilution of R_{18}. The R_{18} fluorescence intensity was measured at room temperature and is proportional to the amount of virus in the sample.

Susceptibility of HA to proteinase K in the intact virus. 200 μg virus in 0.5 ml PBS was pretreated at 37°C at neutral pH, at pH 5.0 and at pH 5.0 in the presence of 2.5 mg/ml DS8. After reneutralizing the samples were incubated with 100 μg Proteinase K (Boehringer Mannheim, Indianapolis, ID) at 37°C for 30 min [13]. The reaction was terminated by addition of 1 mM PMSF. The samples were then precipitated with 10% trichloroacetic acid for 60 min on ice. The precipitate was centrifuged and washed twice with cold ethanol. The pellet was boiled for 15 min in a reducing buffer containing 0.4% SDS, 5 mM Tris, 20 mM dithiothreitol, and 4% glycerol (pH 6.8). The samples were run on a 12% SDS-polyacryamide gel and stained with Coomassie blue.

Results

The influence of dextran sulfate on the fusion between influenza virus and RBC is shown in Fig. 1. The fusion was monitored by the R_{18} fluorescence assay [12]. The lipophilic dye R_{18} is incorporated in the virus membrane at self-quenching concentrations. Upon dye dilution during fusion with the unlabeled RBC membrane the self-quenching decreases and thus the fluorescence intensity increases. To induce fusion, microliter amounts of the virus-RBC complexes were added to PBS at pH 5.0 and 37°C containing different concentrations of dextran sulfate. A clear inhibition of fusion by dextran sulfate can be observed. At 0.5 mg/ml DS500 the fusion was totally blocked. The DS8 molecule with lower molecular weight shows also a significant inhibitory effect, but the inhibition seems to be little weaker than for DS500.

To investigate the influence of dextran sulfate on the HA-mediated cell-RBC fusion, red blood cells were labelled with R_{18} and then bound to GP4F cells at neutral pH. These complexes were added to buffer (pH 5) containing different amounts of dextran sulfate (and Ca^{2+}) and the fusion was followed by the increase of the fluorescence (Fig. 2). In the absence of calcium an increased delay time (time between low pH treatment and onset of fusion) was observed but the extent of fusion was less influenced by adding dextran sulfate. However in the presence of calcium the extent of fusion was strongly decreased, and at high dextran sulfate and Ca^{2+} concentrations the fusion was completely inhibited. Control experiments (without dextran sulfate) indicated that there was no influence of Ca^{2+} itself on the fusion between influenza virus or GP4F cells with RBC (not shown).

To determine if the virus-RBC and the RBC-GP4F cell complexes were dissociated by addition of dextran sulfate we observed the complexes by fluorescence microscopy (not shown). The bound R_{18}-labeled virus were detected by punctate fluorescence on the RBC surface, whereas labeled RBC bound to GP4F cells could be directly visualized and counted. No significant

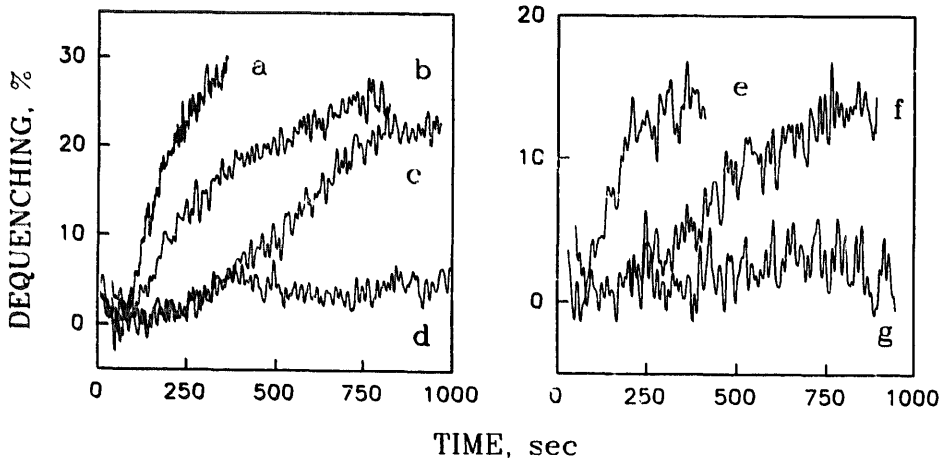

Fig. 2. Influence of DS500 (left) and DS8 (right) on the fusion between RBC and GP4F cells. Fusion was initiated by adding the prebound cell complexes to PBS at pH 5.0 and 37°C. (Left: a, control; b, 0.005 mg/ml DS500; c, 0.5 mg/ml DS500; d, 0.5 mg/ml DS500, 2 mM Ca^{2+}; right: e, control; f, 0.5 mg/ml DS8; g, 0.5 mg/ml DS8, 2 mM Ca^{2+}).

difference after addition of dextran sulfate was found indicating that the prebinding was not disturbed by dextran sulfate and the complexes once formed were stable. Because this visual judgement is not very exact for the binding of virus to RBC control experiments were performed by measuring the R_{18} fluorescence of the bound virus. Virus was prebound to RBC (in the absence of dextran sulfate) and then unbound virus was removed by washing the RBC. Then the cell-virus complexes were added to dextran sulfate containing buffer at 37°C and incubated for 10 min. RBC were spun down and washed and the R_{18} fluorescence of the bound virus (after Triton X-100 addition) was estimated. No difference was found between the control and dextran sulfate-treated RBC-virus complexes, i.e. dextran sulfate does not cause desorption of bound virus (data not shown).

The effect of dextran sulfate on the binding step when virus and GP4F cells, respectively, were incubated with RBC (contrary to the influence of dextran sulfate on the association of already bound complexes as described above) is shown in Table I. The amount of bound virus was estimated by the fluorescence intensity of the R_{18}-labeled virus-RBC complexes after total dequenching by Triton, whereas the average number of bound RBC to GP4F cells was directly counted in the microscope. Without Ca^{2+} no inhibition of binding of virus to RBC occurred. Only a slight decrease of the binding of RBC to GP4F cells by the high molecular weight DS500 was observed. Ca^{2+} slightly increased the binding of virus and GP4F cells with RBC which was partially reversed in the presence of dextran sulfate.

TABLE I

Influence of dextran sulfate on the binding of influenza virus to RBC and of RBC to GP4F cells at pH 7.4 and room temperature

	Virus bound to RBC (%) [a]	Bound RBC per GP4F cell [b]
Control	100 ± 4	1.5 ± 0.2
0.5 mg/ml DS8	98 ± 5	1.5 ± 0.3
0.5 mg/ml DS500	99 ± 5	1.2 ± 0.2
2 mM Ca^{2+}	142 ± 9	1.9 ± 0.3
2 mM Ca^{2+}, 0.5 mg/ml DS8	115 ± 5	1.8 ± 0.3
2 mM Ca^{2+}, 0.5 mg/ml DS500	93 ± 7	1.3 ± 0.3

[a] The extent of binding was estimated from the R_{18} fluorescence intensity (after total dequenching with Triton X-100) of bound virus. The fluorescence of the control (without Ca^{2+} and dextran sulfate) was set to 100%. The values represent the averages of three fluorescence measurements of different samples.

[b] Bound RBC to attached GP4F cells were counted in a microscope. The values represent the averages of five countings of the number of RBC bound to 50 GP4F cells.

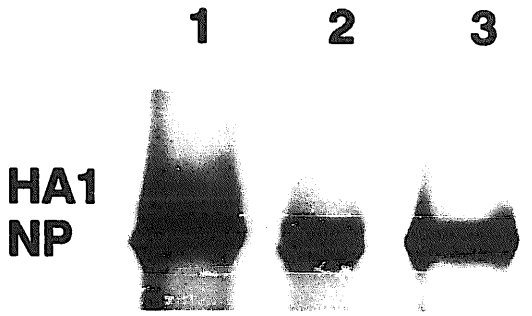

Fig. 3. Effect of acid pretreatment on the susceptibility of influenza virus to digestion by proteinase K in the presence and absence of dextran sulfate. Digestion is indicated by the disappearance of the HA1 band on the 12% SDS-polyacrylamide gel stained with Coomassie blue. Lanes: 1, virus preincubated at pH 7.4, 37°C; 2, virus preincubated at pH 5.0, 37°C; 3, virus preincubated in the presence of 2.5 mg/ml DS8 at pH 5.0, 37°C. Abbreviations: HA, hemagglutinin; NP, nucleoprotein.

The neutral conformation of HA is resistant to proteolytic digestion by trypsin or proteinase K [15,16]. The pH induced conformational change of HA which is associated with the exposure of the fusion peptide converts the protein to a protease sensitive form [15,16]. We used the proteolytic digestion by proteinase K [15] as a method to examine if this conformational change of the viral HA is affected by dextran sulfate. The digestion of low pH treated virus with proteinase K causes nonspecific degradation of the HA peptide that can be seen by the disappearance of the HA1 band in the SDS-PAGE under reducing conditions (Fig. 3). In the presence of DS8 no difference was found, i.e., HA1 is also degraded. A higher DS8 concentration was chosen than for the binding and fusion assays in order to compensate for the higher amount of protein used in this experiment. The use of DS500 caused technical problems: presumably due to the high charge and size of the macromolecule the protein bands in the SDS-PAGE are strongly disturbed and appeared much weaker and smeared. To overcome this problem we tried to separate the DS500 from the virus on a Bio-Gel A-150m (Bio-Rad, Richmond, CA) after the incubation at low pH. The results could be much improved, but still some smearing of the bands occurred and furthermore it is difficult to load exactly the same protein amount on the gel after the purification step. A judgement is thus difficult and the result was not as convincing as for DS8. But it seemed that HA was also digested when the virus was preincubated at pH 5 with DS500 (not shown). The HA1 and HA2 bands disappeared and breakdown products could be detected.

The fusion of RBC with GP4F cells does not start immediately after lowering the pH, but is preceded by a delay time of about 20–60 s at 37°C [11,17]. To examine, whether dextran sulfate acts at this early pre-fusion state DS500 was added at different times between lowering the pH and the onset of fusion (in

the presence of Ca^{2+}). The effect on fusion was more pronounced at the shorter time intervals (Fig. 4) indicating that the presence of dextran sulfate during early steps is necessary to cause inhibition of fusion.

It has been previously shown that during the pre-fusion state HA can be committed to subsequent fusion at neutral pH [11,17]. In other words, only the initial events require low pH whereas subsequent steps occur even at neutral pH. The prefusion state can be attained at 0°C by lowering the pH of prebound virus-cell or cell-cell complexes after which fusion can occur by incubating at neutral pH at 37°C (Clague et al., unpublished data). By using this experimental approach it was possible to investigate if low pH requiring steps are disturbed by dextran sulfate. When RBC-GP4F cell complexes were exposed at pH 5, 0°C, fusion was very slow with a lag time larger than 25 min (not shown) in accordance with results in Refs. 14 and 19 for intact virus.

When the RBC-GP4F cell complexes were preincubated for 15 min at pH 5, 0°C in 100 μl sample volume, followed by adding to 2 ml PBS at pH 7.4, 37°C a rapid increase in fluorescence was observed (curve A in Fig. 5, the pH in the mixture was sligthly shifted to 7.25). To expose the cells to dextran sulfate only during the pre-fusion state, the sample was incubated for 15 min at pH 5.0, 0°C in the presence of 0.5 mg/ml DS500, 2 mM Ca^{2+}, followed by washing with PBS (pH 7.4, 0°C) to remove dextran sulfate. Then the pellet was resuspended in 100 μl PBS (pH 7.4, 0°C) and added to 2 ml PBS at pH 7.4, 37°C (Fig. 5, curve B). The fusion rate was somewhat lower but the extent was not influenced. This indicates that all steps in the pre-fusion state that require low pH occurred even in the presence of dextran sulfate and are thus not inhibited. In order to examine if dextran sulfate inhibits the fusion at neutral pH after commitment, 100 μl sample was preincubated at pH 5, 0°C and after 15 min added to buffer at pH 7.4, 37°C containing 0.5 mg/ml DS500, 2 mM Ca^{2+} (Fig. 5, curve C). In agreement with the data in Fig. 4, no inhibition occurred since the presence of dextran sulfate at late stages is ineffective. In summary, dextran sulfate must be present during the pre-fusion state as well remain bound at later stages to inhibit fusion.

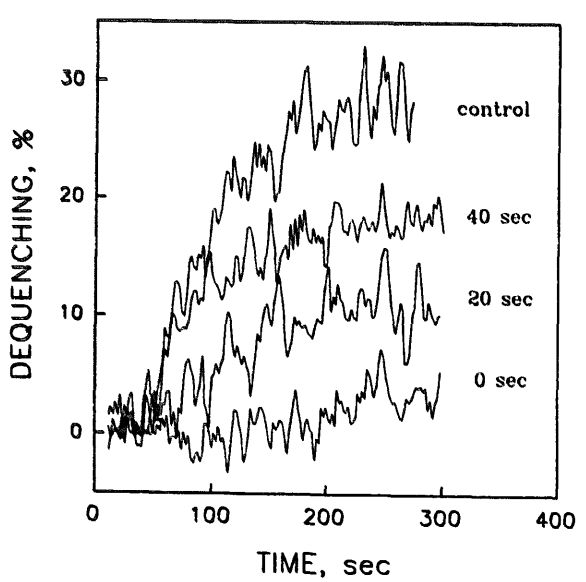

Fig. 4. Influence of the time interval between inducing the fusion by addition of RBC-GP4F cell complexes to buffer at pH 5.0, 37°C and the addition of 0.5 mg/ml DS500 in the presence of 2 mM Ca^{2+}.

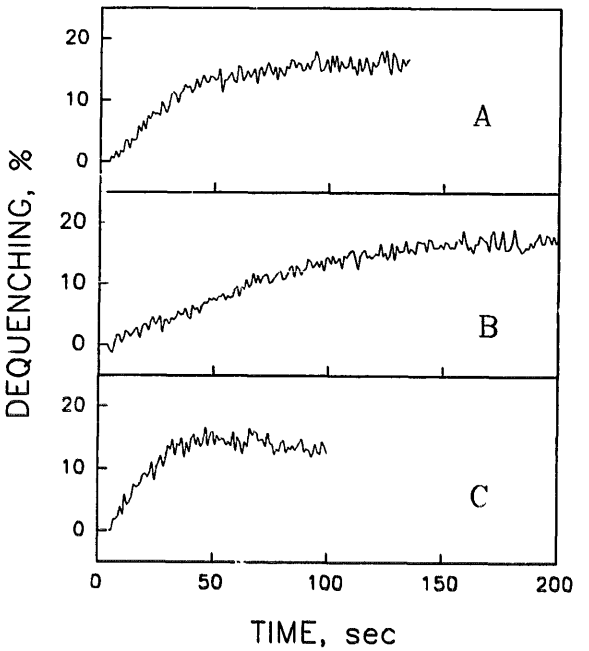

Fig. 5. Effect of dextran sulfate on commitment of RBC-GP4F cell complexes at low pH and low temperature to fusion at neutral pH, 37°C. (A) 15 min pH 5.0, 0°C; at $t = 0$ pH 7.4, 37°C. (B) 15 min pH 5.0, 0°C, 0.5 mg/ml DS500, 2 mM Ca^{2+}; two times washing (with PBS, pH 7.4, 0°C); at $t = 0$ pH 7.4, 37°C. (C) 15 min pH 5.0, 0°C; at $t = 0$ pH 7.4, 37°C, 0.5 mg/ml DS500, 2 mM Ca^{2+}.

Discussion

The infectivity of enveloped viruses is known to be inhibited by dextran sulfate. This effect is mostly explained by blocking of the virus binding to the host cell. So far no detailed studies on the molecular mechanisms of the action of the polymer have been done. The better understanding of these processes may lead to new approaches in the development of antiviral drugs. In a recent paper the inhibitory effect of dextran sulfate on the fusion of influenza virus with DOPC/cholesterol liposomes was described [8]. It was concluded that dextran sulfate inactivates the hemagglutinin by binding to the viral protein and interfering with the low pH induced conformational change. However, no clear evidence was given to support this idea. Furthermore influenza virus can not be prebound to zwitterionic liposomes. Thus it can be not decided if

the specific binding step or the fusion is influenced by dextran sulfate.

In the present paper we investigated the action of dextran sulfate on the low-pH-induced fusion of red blood cells with influenza virus and with GP4F cells expressing the influenza spike protein on the surface. The binding and fusion were dissected to investigate the influence of dextran sulfate on both steps separately. Furthermore the influence of dextran sulfate on different steps during the fusion reaction that lead to the membrane merging was examined.

The prebinding of the virus or GP4F cells to red blood cells at neutral pH was only little or not at all inhibited by dextran sulfate Table I). In contrast to the work with liposomes [8] the binding was due to specific interactions between the binding sites on HA and sialoglycolipids/proteins on the target membrane [19] The increase in binding in the presence of Ca^{2+} is presumably due to nonspecific electrostatic interactions, which can be counteracted by dextran sulfate.

A strong inhibition of fusion with RBC by dextran sulfate was found for both influenza virus, and in the presence of Ca^{2+} for GP4F cells (Figs. 1 and 2). This inhibition of fusion was much stronger than the inhibition of binding mentioned above. Presumably, the influence of dextran sulfate is mediated by electrostatic binding of the negatively charged macromolecule to the viral fusion protein. FITC-dextran sulfate bound mainly to GP4F cells and only weakly to RBC as visualized by fluorescence microscopy (not shown) indicating that the effect of dextran sulfate is not caused by binding of the macromolecule to the target membrane. The influence of Ca^{2+} is less clear. Ca^{2+} increased the inhibitory effect of dextran sulfate on the fusion between RBC and GP4F cells. This is possibly due to an increased binding affinity of dextran sulfate caused by the divalent cation that can serve a bridging function as found for other systems [20]. Ca^{2+} did not significantly influence the inhibition of fusion of influenza virus with erythrocytes (data not shown).

It has been previously shown that pH-triggering of HA results in a multi-step series of events eventually leading to fusion [21]. At low pH a very fast conformational change occurs which results mainly in the exposure of the hydrophobic fusion peptide [22] and renders HA into a proteinase-sensitive form [15,16]. This conformational change was not affected by DS8 because low pH treatment of virus in the absence as well in the presence of DS8 made HA susceptible to proteinase K (Fig. 3). DS500 seems also not to interfere with the conformational change at low pH but the results are less clear for technical problems (as described in the results section in detail). Under several conditions (low temperature, low surface concentration of HA expressed in cells) a lag time before the onset of the membrane merging is observed [11,18]. During this pre-fusion state further structural rearrangements occur that lead to the formation of fusion-competent complexes. These steps are likely to represent the insertion of the fusion peptide into the target membrane [23], aggregation of the HA molecules in the viral membrane [9] and proper arrangements of HA to form fusion pores [24,25]. Some of these steps also require low pH (the low pH induced exposure of the fusion peptide is not sufficient for fusion [26]).

Our results indicate that dextran sulfate needs to be present throughout the whole reaction to be inhibitory (Figs. 4 and 5). However, the low pH requiring steps seem to be not inhibited by dextran sulfate (see Figs. 3 and 5). Once the system has undergone sufficient structural rearrangements the addition of dextran sulfate is not effective (Figs. 4 and 5). One may speculate that the effect of dextran sulfate is caused by interfering with steps after low pH-requiring conformational changes leading to formation of the 'pre-fusion complex'. When these steps have occurred in the absence of dextran sulfate the complex is presumably in such a structural state (e.g., association of HA with each other and with the target membrane) that permits no longer effective access of dextran sulfate.

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