Biochimica et Biophysica Acta, 1148 (1993) 1–6

BBAMEM 75946

Effect of glycosaminoglycans and PEG on fusion of Sendai virus with phosphatidylserine vesicles

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(Received 29 December 1992)

Key words: Sendai virus; Membrane fusion; Liposome; Glycosaminoglycan; Dextran sulfate; Poly(ethylene glycol)

The fusion of Sendai virus with phosphatidylserine vesicles was monitored by a pyrene-phosphatidylcholine fluorescence assay. A strong influence of pH and ionic strength on the extent of fusion was observed. The negatively-charged polymers (dextran sulfate, heparin and chondroitin sulfate) inhibited the ability of the viruses to fuse with the liposomes. The extent of inhibition, for a given amount (w/v) of the polymers, was the greatest for dextran sulfate followed by heparine and chondroitin sulfate. The extent of inhibition depended on the pH and ionic strength of the solution; the lower the pH of the solution, the more effective the fusion inhibition by the polymers. The molecular weight of dextran sulfate (DS) influenced the inhibition effect, i.e., DS with higher molecular weight exhibited a stronger inhibition effect. The presence of sodium sulfate, even in excess concentration, had no inhibitory effect on fusion. On the other hand, PEG had an opposite effect on fusion compared to the negatively-charged polymers, and it decreased their inhibition effect when both were present in the same media. It is concluded that the inhibition of the fusion activity of Sendai virus results from the adherence of negatively-charged polymers to the virus surface preventing close contacts between the virus and liposome surface.

Introduction

Fusion of liposomes has been widely used as a model for studying the molecular mechanism of membrane fusion (for a review, see Ref. 19). Lipid membrane fusion can be induced by the addition of multivalent cations, such as Ca^{2+} and Mg^{2+} [16,28], by proteins [6] or by macromolecules, such as poly(ethylene glycol) [20,29] as well as cationic (e.g., polylysine) [23] and anionic polymers (e.g., dextran sulfate) [3] or by different types of viruses [7,25]. The molecular mechanism of protein-induced membrane fusion seems to be different from that of ion-induced membrane fusion. A typical example for protein-induced membrane fusion may be seen in the virus-host membrane fusion systems. The elucidation of virus-membrane fusion processes is of special interest because of their clinical relevance.

Sendai virus contains two major surface proteins: HN protein, which plays a role in virion adsorption on the cell surface by binding to a specific receptor on a host cell surface, and F protein, which is the fusion protein responsible for fusion of viral envelope with host cell membranes. For Sendai virus-cell fusion systems, the presence of both proteins (HN and F) seems necessary. On the other hand, for the fusion of acidic phospholipid membranes and Sendai virions, only the presence of HN in viral envelope seems to be sufficient to induce fusion [1]. The latter fusion process is rather unspecific when compared to the virus-cell fusion processes [13]. However, the use of liposomes instead of host cells provides a system which is simple enough for experimental manipulations and for experiments under definite conditions.

The fusion of viruses with liposomes depends on the solution properties (temperature, pH, ionic strength, etc.) [13], as well as on the types of viruses and the composition of liposomes [9]. These dependences are mainly due to the nature of viral membrane proteins. The amino-acid sequence of the N-terminus of the F1 protein was described by Gething et al. [5]. The ionization of the viral glycoproteins of Sendai virus takes

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Abbreviations: PS, phosphatidylserine; Pyr-PC, 3-palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine; PEG, poly(ethylene glycol); HIV, human immunodeficiency virus; DS, dextran sulfate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SUV, small unilamellar vesicles; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; GAG, glycosaminoglycan.

place in a broad pH range [24]. From the amino-acid composition of the HN protein it is possible that the protein possesses an overall positive charge at neutral pH.

Glycosaminoglycans (GAGs) comprise negatively-charged macromolecules such as heparin and chondroitin sulfate which are important components of the extracellular matrix. Dextran sulfate (DS) is similar in structure and has often been used as a model compound for GAG. These polymers are known to bind especially to positively charged residues of proteins, such as lipoproteins [14] and liposomes containing stearylamine [3]. Dextran sulfate influences the adsorption of viruses on cells [4]. It is also able to suppress the binding of HIV to CD4 cells [15] and it is used against HIV-1 infection in vitro [26]. It has recently been reported that dextran sulfate suppresses the fusion of Sendai virus [21] and influenza virus [31] with phospholipid vesicles.

Poly(ethylene glycol) was found to enhance the fusion of Sendai virus with liposomes, as well as erythrocyte ghost membranes, probably due to the ability of PEG to dehydrate the local contact area between viral and target membranes [12,17]. It is interesting to investigate how the virus-induced membrane fusion is affected by the presence of both types of polymers (i.e., anionic polymers and PEG).

In this study, we examined the effect of DS, heparin, chondroitin sulfate and PEG on Sendai virus-PS vesicle fusion under various experimental conditions: as a function of concentration and molecular weight of the polymers, and as a function of pH and ionic strength of the suspension media. A prelimimary report about the influence of DS on the fusion of Sendai virus with PS vesicles has been given recently [31]. A new aspect of this study was to use dextran sulfate of different molecular weight and other sulfated polymers (heparin and chondroitin sulfate), in order to examine the effects of molecular weight and negative SO_3^- charge density of the polymers on the inhibition of virus induced membrane fusion. We also report here the effects of ionic strength of the environmental media, as well as the simultaneous presence of fusion inhibitory and enhancing polymers on fusion.

Materials and Methods

Bovine-PS and egg-PC from Avanti were used without further purification. The fluorescent probe 3-palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholin e (Pyr-PC) was obtained from Molecular Probes. Dextran sulfate MW 8000 and 40 000 were purchased from ICN, and both heparin (144 U/mg, MW 15 000) from bovine lung and chondroitin sulfate C (MW 60 000) from shark cartilage were obtained from Calbiochem. Poly(ethylene glycol), MW 6000 (PEG 6000), was a product from Fluka and was used without further purification.

Buffer solutions used were: 10 mM citrate for pH 4 and 5, 10 mM Mes for pH 6 and 10 mM Hepes for pH 7 and 8.

All solutions contained 0.05 mM EDTA and 0.1 M NaCl, unless otherwise stated. Small unilamellar vesicles (SUV) were prepared by dissolving bovine-PS and Pyr-PC (10 mol%) in chloroform, drying under a stream of nitrogen, and adding an appropriate buffer solution, followed by gentle vortexing (5 min). Then, the suspension was sonicated for 40 min. Cantell strain Sendai virus, grown in fertilized chicken eggs was isolated from allantoic fluid, purified according to the published method [10] and suspended in 0.1 M phosphate buffer (pH 7.0). Stock virus was aliquoted and stored at -40°C.

Fluorescence measurements were carried out on a Perkin-Elmer LS 5 spectrofluorimeter equipped with a thermostat unit. All measurements were done at 37°C. The excitation wavelength was 340 nm and the fluorescence emission was measured in the range of 350–500 nm. The emission maximum of the pyrene excimer (E) occurred at 460 nm and that of the monomer (M) at 376 nm. In our measurements, 50 μl of the virus stock solution (2 mg viral protein/ ml of 10 mM Hepes, 0.15 M NaCl (pH 7.0)) was added to 2 ml of the prewarmed buffer solution (37°C) containing 2.5 μM PS/Pyr-PC liposomes.

According to Amselem et al. [1], a probe dilution factor ($D_p - 1$) was calculated (Eqn. 1) from the excimer/monomer ratios (E/M) measured at the time of mixing of virus and liposomes $(E/M)_0$ and at time t after mixing $(E/M)_t$.

($D_p - 1$) is given by the following equation:

$$(D_p - 1) = [(E/M)_0 - (E/M)_t]/(E/M)_t \quad (1)$$

Eqn. 1 can be interpreted following the approach of Nir et al. [18] as:

$$(D_p - 1) = V_F/L \quad (2)$$

where V_F represents the concentration of the fused viral lipid and L the total lipid of the vesicles.

Results

Fusion of Sendai virus with phosphatidylserine (containing 10 mol% Pyr-PC) vesicles was measured by monitoring the pyrene fluorescence. In Fig. 1, the probe dilution factors ($D_p - 1$) are plotted against time at different pH values. The increase in ($D_p - 1$) indicates the dilution of the fluorescence probe which corresponds to fusion of labelled PS vesicles with unlabelled virus. In the absence of virus, ($D_p - 1$) of the

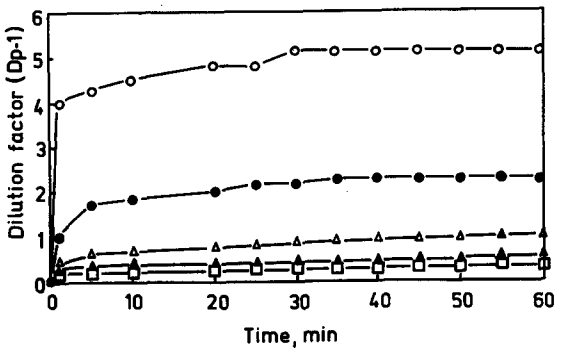

Fig. 1. Time-dependence of $(D_p - 1)$ of PS/Pyr-PC SUV after addition of Sendai virus. The pH of the solutions was 8 (□), 7 (▲), 6 (△), 5 (●) or 4 (○). 0.1 M NaCl was used in all experiments. Temperature was 37°C.

same vesicle preparation was time-independent, which was in accordance with the measurements done by Amselem et al. [1,2]. The increase in $(D_p - 1)$ was dependent on the pH of the solution; the more acidic the solution, the greater was the magnitude of increase in $(D_p - 1)$, i.e., the fusion extent was higher at low pH.

Application of Eqn. 2 using 2.5 μM vesicle PL concentration and 12.5 μM virus PL concentration gives at pH 4.0 about 100% fusion, for pH 5.0 about 40% fusion, for pH 6.0 20% fusion and for pH 7 and 8 less than 10% fusion.

After adding Sendai virus, a small decrease of the E/M ratio was observed because the increase in turbidity and light scattering contributed to the increase of the base-line in the range of the monomer signal. This contribution was corrected by subtracting the contribution due to light scattering from the total fluorescence signal. The spectrum of scattered light was determined by use of unlabelled PS liposomes for each case of the control experiment (labelled liposomes) and virus-liposome experiment (virus + labelled liposomes) for the fluorescence experiment.

It should be noted that we observed some degree of changes in E/M during the incubation procedure at 4°C of Sendai virus with PS liposomes, especially at low pH values. Therefore, in order to monitor exactly the time-course of probe dilution in the process of fusion, all experiments were carried out without preincubation at low temperature.

In Fig. 2, the influences of different anionic macromolecules (heparin, dextran sulfate 8000 and 40000, chondroitin sulfate C) on the dilution factor $(D_p - 1)$ of PS/Pyr-PC SUV after addition of Sendai virus at pH 5 and pH 7.4 are given. $(D_p - 1)$ was measured as follows: A given volume of the virus solution (50 μl) was transferred to the prewarmed buffer solution (37°C) either or not containing an appropriate amount of anionic macromolecules. The samples were incubated for 5 min at 37°C. After incubation, PS/Pyr-PC liposomes (50 μl) were added, and the spectrum was recorded immediately.

By comparison of the time-courses of fluorescence of samples in the presence of polymers (1 mg/ml) and in the absence of polymer (control) in the media (Fig. 2), it is clearly seen that all polymers inhibited the fusion process, although the extent of inhibition was different for different polymers. The inhibition effect increases in the order CSC < DS 8000 < heparin < DS 40000 in the polymer concentration range below 0.1 mg/ml. In this experiment, the fusion of the control according to Eqn. 2 at pH 5.0 was about 60% (for pH 7.4 12%), for 1 mg/ml CSC about 30% (pH 7.4 12%), for 1 mg/ml DS 8000, DS 40000 and heparin about 10% (pH 7.4 about 7%). At pH 7.4, the fusion extent in the absence of the anionic polymers was much smaller than that at pH 5.0 and the absolute changes due to the presence of anionic macromolecules were found to be less pronounced at pH 7.4. At this pH, heparin, dextran sulfate 8000 and 40000 exhibited a similar degree of inhibition of fusion. The effect of CSC at pH 7.4 was negligable.

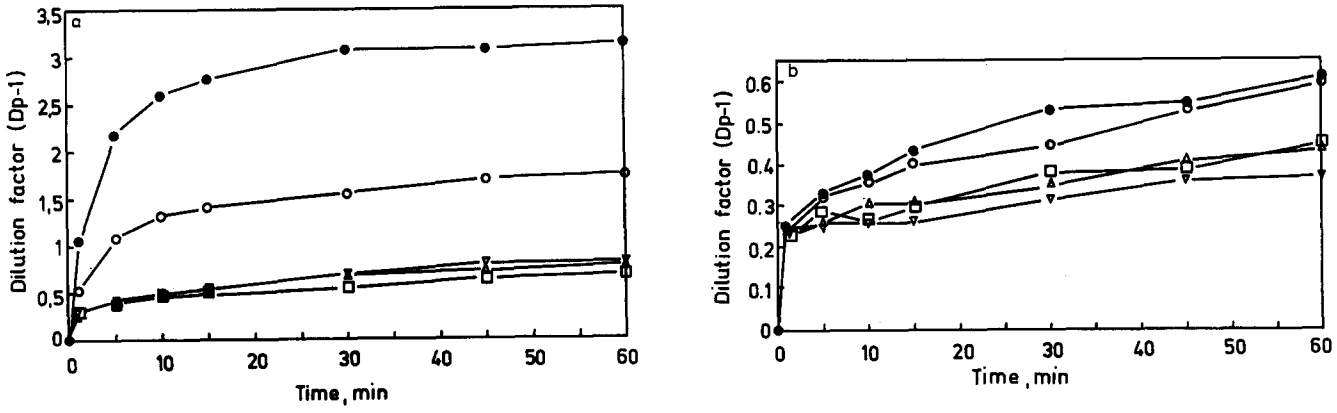

Fig. 2. Time-dependence of $(D_p - 1)$ of PS/Pyr-PC SUV after addition of Sendai virus at pH 5 (a) and pH 7.4 (b) in the presence of (□), 1 mg/ml dextran sulfate 40000; (▽), 1 mg/ml dextran sulfate 8000; (△), 1 mg/ml heparin and (○), 1 mg/ml chondroitin sulfate C. (●), Control.

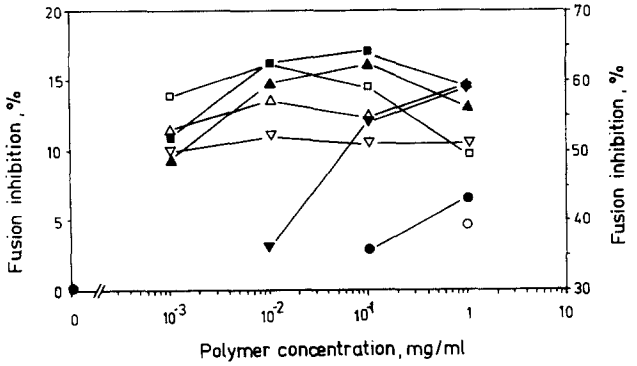

Fig. 3. Inhibition of Sendai virus-PS fusion (determined from the E/M value after 30 min, the corresponding E/M value for the control was set to be 100%) by different concentrations of DS 8000 (▽), DS 40000 (□), CSC (○) and heparin (△) for pH 5 (filled symbols and right ordinate) and pH 7.4 (open symbols and left ordinate).

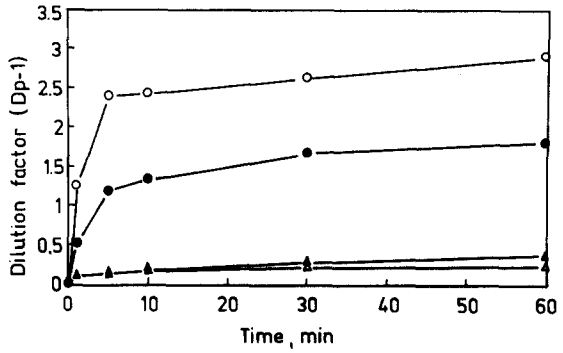

Fig. 4. Time-dependence of $(D_p - 1)$ of PS/Pyr-PC SUV after addition of Sendai virus at pH 5.0 in the absence (○, ●) and presence of 0.1 mg/ml DS 40000 (△, ▲). The open symbols refer to 0.01 M NaCl, the filled ones to 0.14 M NaCl.

In order to determine a possible difference between the different orders of incubation of the samples, the following case was also examined; we incubated heparin, dextran sulfate (MW 8000 and 40000) or chondroitin sulfate C with PS/Pyr-PC liposomes first for 5 min at 37°C, and then Sendai virus was added. The results obtained for this preincubation did not indicate any differences from those given in Fig. 2, where Sendai virus was preincubated with the anionic polymers first, and then lipid vesicles were added later. Simultaneous addition of Sendai virus and PS SUV to the buffer solution containing 1 mg/ml DS 40000 suppressed the fusion to the same amount as given in Fig. 2. Also, if we added DS to the PS/Sendai virus solution after 2 min, the decrease in E/M was stopped at this point.

While in Fig. 2 the inhibiton effect of DS, heparin and CSC at one distinct concentration (1 mg/ml) is shown, the effect of the concentration of the anionic polymers on the fusion of Sendai virus with PS liposomes is given in Fig. 3. The change in E/M in the time-range 0–30 min. of a control sample was set to be 100% fusion. Then, the change in E/M (0–30 min) of the samples containing anionic polymers was calculated, relative to the corresponding E/M value for 100% fusion of the control sample. The difference from 100% fusion was used as fusion inhibition in Fig. 3. At pH 5, the extent of inhibition by DS 40000 and heparin was about 60% and did not depend on concentration in the concentration range of 10^{-3}–1 mg/ml, whereas for DS 8000, a strong dependence on concentration was observed. CSC inhibited fusion of Sendai virus with PS liposomes at higher concentrations (10^{-1}–1 mg/ml) for about 30% to 40%. At pH 7.4, the extent of inhibition for DS 8000, 40000 and heparin was about 15% in the concentration range of 10^{-3}–1 mg/ml. The inhibitory effect of CSC was very low and occurred only at high concentrations (1 mg/ml).

In order to check the influence of ionic strength of the solution on Sendai virus-PS/Pyr-PC SUV fusion, we determined $(D_p - 1)$ at pH 7.4 and pH 5.0 in 0.14 and 0.01 M NaCl. The results are given in Figs. 4 and 5. The reduction of ionic strength led to a greater extent of fusion both at both pH 7.4 and 5.0. In the presence of 0.1 mg DS 40000/ml, the extent of fusion was suppressed in a different manner for both 0.01 and 0.14 M NaCl at pH 5.0 and 7.4. Using the same procedure for calculation of inhibition by 0.1 mg DS 40000/ml as applied for the calculation of data given in Fig. 3 the following values were obtained: For pH 5.0, 10 mM NaCl an inhibition of 75.2%, for pH 5.0, 140 mM NaCl 64.8% was determined, respectively. In the solution of pH 7.4 and 10 mM NaCl, the inhibition by DS 40000 was 41%, whereas in 140 mM NaCl an inhibition of 8% was calculated.

Because of the obvious electrostatic nature of the interaction between DS and the virus, we tried to obtain more information about the necessary 'properties' of the negatively-charged molecules. DS, heparin and chondroitin sulfate C contain negatively-charged SO_3^- groups. Addition of high concentrations of

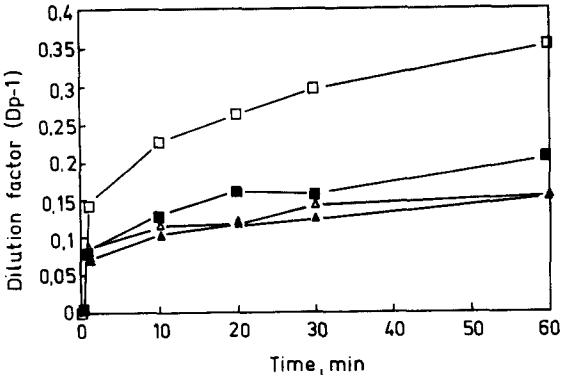

Fig. 5. Time-dependence of $(D_p - 1)$ of PS/Pyr-PC SUV after addition of Sendai virus at pH 7.4 in the absence (□, ■) and presence of 0.1 mg/ml DS 40000 (△, ▲). The open symbols refer to 0.01 M NaCl, the filled ones to 0.14 M NaCl.

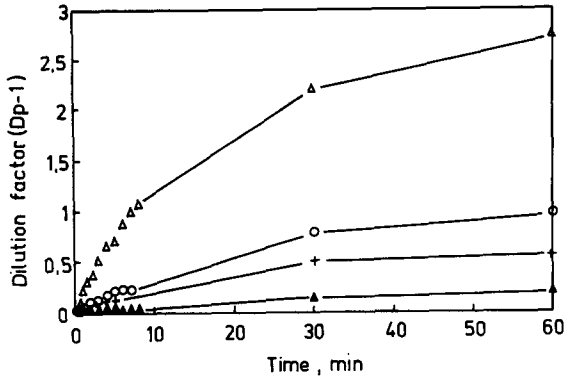

Fig. 6. Effect of PEG 6000 and DS 40000 on the fusion of Sendai virus with phosphatidylserine liposomes. (buffer; 5 mM Hepes, 0.1 M NaCl (pH 6.0)) (○), without polymer; (△), with PEG 6000 (8% (w/w)); (▲), with DS 40000 (1 mg/ml); (+), with PEG 6000 (8% (w/w)) and DS 40000 (1 mg/ml).

Na_2SO_4 instead of NaCl (up to 0.1 mol/l) did not lead to any inhibition of the fusion between Sendai virus and PS/Pyr-PC liposomes at pH 5.0 (data not shown).

When the sample medium contained 8% PEG 6000 (w/w), a large increase in $(D_p - 1)$ occurred, as compared to those obtained in polymer-free medium, as shown in Fig. 6. An increase of the rate and extent of fusion of Sendai virus with phosphatidylserine liposomes in the presence of PEG was described by Nir et al. [17]. Our measurements confirmed these findings. As described above the change in $(D_p - 1)$ was much reduced in the presence of 1 mg/ml DS 40000, indicating an inhibition of fusion (also see Fig. 6). When 8% PEG 6000 (w/w), as well as 1 mg/ml DS 40000 were in the medium, the fusion extent was higher compared to the sample containing only DS 40000, but it was still lower than that observed without any polymer. This experiment shows that the inhibiting effect of dextran sulfate is preserved in the presence of a component enhancing virus-liposome fusion.

Discussion

For monitoring virus-liposome fusion, a fusion assay, which does not use charged probe molecules, was chosen. The charged probe molecules, as R_{18}, usually employed for virus-cell fusion, can act as binding sites of the negatively charged polymers, whereas Pyr-PC does not interact with these polymers. It has been shown that the anionic macromolecules used do not interact with negatively-charged phospholipid membranes and neutral liposomes [3]. The binding of these macromolecules requires positively-charged residues as binding partners. This has been shown by using positively charged liposomes [27] or lipoproteins [14]. The latter system may be comparable to virus with respect to the surface structure and particle size. In both systems the surfaces consist of a phospholipid layer with a small number of proteins incorporated. The interaction of lipoproteins with the polymers used in this paper was studied by Krumbiegel et al. [14]. All lipoproteins (LDL, HDL) exhibit an overall negative surface charge at pH 7.4, but they are able to bind glycosaminoglycans, such as heparin, chondroitin sulfate or DS. Sendai virus exhibits a negative surface charge under physiological conditions as was measured by microelectrophoresis [7,22]. Looking at the amino-acid composition of the HN and F proteins, there are several positively-charged residues as potential binding sites for DS, heparin and CSC.

Decreasing the pH to 5.0 the average negative surface charge is reduced and an increased binding of anionic polymers is possible. Taking into account that the isoelectric points of the viral HN and F glycoproteins are expected to be 6.5 and 4.9, respectively [24], the average surface charge at pH 5 tends to be zero or positive. Because of the greater electrostatic attractions the binding of anionic polymers is then higher. In fact an aggregation of Sendai virus by dextran sulfate was found for pH values lower than 7, which can be explained by a binding of the polymer to the virus surface and the formation of polymer bridges between virus particles [21]. One can claim that the DS-induced aggregation of the virus is the reason of their lower fusion ability, but this explanation is contradicted by our observation that PEG induces an aggregation of the virus, but the virus activity is increased.

At anionic polymer concentrations lower than 0.1 mg/ml, the order of effectiveness of the polymers to suppress fusion was DS 40000 > Heparin > DS 8000 > Chondroitin sulfate C at the same amount (w/v) of the polymers applied to the sample media, regardless of the pH.

Considering that the molecular weights of heparin and CSC used in this work were 15000 and 60000, respectively, and also the number of charged groups (SO_3^-) per unit monomer of these polymers are 3 for DS, about 2 for heparin and 1 for CSC, the effectiveness of the polymer to suppress fusion appeared to be positively correlated with the negative charge (SO_3^-) density on the polymer, as well as the size (molecular weight) of the polymer.

There was also a concentration-dependence on the effectiveness of the polymer in the range of concentrations lower than 0.1 mg/ml; the higher the polymer concentration, the more effective in its inhibition on fusion. This was clearly seen for the experimental results done at lower pH than neutral pH. This finding agrees with those found in the Sendai virus-erythrocyte fusion system [22]. At polymer concentrations greater than 0.1 mg/ml, the effectivenes of suppression of fusion was either saturated or even slightly reduced.

For the different precipitation behaviour of lipoproteins induced by heparin, DS and chondroitin sulfate it

was argued that for the binding of GAG and DS the charge density of the polymers is essential. Only polymers with high charge density were able to aggregate lipoproteins. In our experimental system this view is supported by the findings that even high concentrations of SO_4^{2-} ions, e.g., 0.1 M Na_2SO_4 do not lead to inhibition of the virus-induced fusion process. It is interesting to note that the ability of the polymers to precipitate lipoproteins coincides with the inhibition effect on the virus-liposome fusion, indicating a strong relation to the extent of binding of the polymer to the virus surface.

It should be noted that we observed some increase in $(D_p - 1)$ for Sendai virus incubated with PS SUV at 4°C; during incubation of the sample at 4°C for 4 min, about 20% of the probe dilution occurred compared with that of the sample transfered to 37°C (without preincubation at 4°C) for the same time period. Because of the physicochemical properties of the pyrene-labelled phospholipid it is unlikely that this strong increase in $(D_p - 1)$ is caused by probe exchange.

It was observed that the rate and the extent of fusion of Sendai virus with PS membranes are increased in the presence of PEG [17], especially the rate constant of fusion. The function of PEG in this process is seen in a close approach of virus and liposome membranes enabling a better penetration of the virus fusion protein into the liposome membrane [12]. As our experiments show (Fig. 6), dextran sulfate still has a strong inhibiting effect on the fusion process in the presence of PEG. The observation that PEG is able to reduce the inhibition effect of dextran sulfate may be explained by opposite actions of these polymers: PEG increases whereas dextran sulfate prevents the close approach of the virus and liposome membranes due to the adherence of this negatively-charged and hydrophilic molecule to the virus surface.

Acknowledgements

This work was supported by the grant U.S. NIH GM 24840 (to S.O.) and DFG Ar 195/1-1 (to K.A.).

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