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Effects of anionic and nonionic polymers on fusion and binding of Sendai virus to human erythrocyte ghosts

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Abstract

Effects of various polymers (dextran sulfate, dextran and polyethylene glycol) on binding and fusion of Sendai virus to target cells were studied by use of fluorescence spectroscopy. Direct binding of dextran sulfate but not dextran to Sendai virus was detected. Anionic and nonionic polymers showed definite effects on segmental motions of the viral envelope proteins. Sendai virus binding to human erythrocyte ghost membranes (HEG) was reduced by dextran sulfate and dextran while the fusion temperature dependence remained unaltered at ≈ 20°C. Nonionic polymer, polyethylene glycol, caused an increase in extent of fusion of Sendai virus with HEG. Segmental motion of viral envelope proteins, determined in terms of anisotropy of fluorescent probes attached to viral surface proteins, exhibited a temperature dependent transition at 20°C by a sharp change from restricted to less restricted motion. In the presence of each of the polymers, this transition was no longer apparent. Since fusion did occur in the presence of all polymers, the temperature dependent characteristic of Sendai virus target cell fusion can be said not to depend on viral surface protein segmental motion. A reasonable and coherent explanation was given for the apparent disparity between the effects of inhibiting and enhancing polymers on fusion and motion of viral proteins.

Keywords: Binding; Fusion; Sendai virus; Viral envelope proteins; Protein mobility

1. Introduction

Sendai virus, a paramyxovirus, delivers the contents of its nucleocapsid into a target cell by way of membrane fusion that is temperature depen-

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dent and protein-mediated. The initiation of membrane fusion events of Sendai virus with target cells or lipid vesicles does not appreciably begin until temperatures above a fusion-threshold temperature (FuT) of 18–20°C are reached (Lee et al., 1983; Klappe et al., 1986; Aroeti et al., 1990).

The envelope of Sendai virus contains two glycoproteins, hemagglutinin/neuraminidase (HN) and fusion (F) protein (Shimizu et al., 1974). The protein-induced membrane fusion mechanism is preceded by a two step binding process that is initiated by the viral HN envelope glycoprotein while the fusion event is initiated by the F protein. The HN protein binds to sialic residues of sialoglycoproteins independently of temperature, providing access to sialic acid residues of gangliosides in the membrane of the target cell.

Stable binding would be considered at the stage of interaction between the target membrane and the virus that leads only to fusion and not dissociation. Several authors (Hoekstra et al., 1985; Klappe et al., 1986; Tsao and Huang, 1986; Haywood and Boyer, 1981; Haywood, 1991) have stated that stable binding must occur before the fusion event and that the HN protein provides this binding. Haywood, 1991 showed that the presence of nonionic polymers (i.e. dextran and ficoll) allowed Sendai virus to stay bound to receptor containing liposomes at 4°C. This effect of the polymers was dependent on the size and concentration of the polymer. Hoekstra et al. (1989) reported a similar binding enhancement result with PEG (at 6% PEG 8k) with a maximal increase in Sendai virus binding to human erythrocyte ghosts (HEG) by 20%. Sendai virus bound at low temperatures to either liposomes or cells can be partly removed by about 30% simply with the addition of warm buffer (Hoekstra and Klappe, 1986). However, since the presence of nonionic polymers is effective in preventing this dissociation (Hoekstra et al., 1989), a more stable binding in the presence of the polymers is hypothesized.

Inhibiting the binding of Sendai virus using anionic polymers (i.e. dextran sulfate), which is thought to bind to the viral HN envelope proteins (Ohki et al., 1992) has been pursued as an anti-viral agent for other viruses such as HIV–1 (Mitsuya et al., 1988; Mbemba et al., 1994). Anionic polymers such as dextran sulfate have been shown to interfere with the fusion of Sendai virus via steric effects as well as by other possible physicochemical factors (Ohki et al., 1992; Zschörnig et al., 1993). Whether dextran sulfate has an effect on the temperature dependence of Sendai virus fusion is interesting since there is direct interaction of the anionic polymer with the surface proteins of the virus. If the temperature dependence of fusion resides in the viral envelope proteins, as has been suggested (Hoekstra et al., 1985), then interference with these proteins may result in a change in the FuT.

So far little work has been done regarding the effect of these polymers on viral glycoproteins. In this paper, we have studied the effect of anionic and nonionic polymers on the temperature dependent fusion of Sendai virus with erythrocyte ghosts and the effect of the polymers on rotational and/or segmental motion of viral glycoproteins with respect to temperature in order to elucidate the interaction mode of the polymers with virus and the nature of temperature dependent fusion.

2. Materials and methods

Octadecyl rhodamine B chloride (R-18), dextran sulfate-fluorescein, 40000 MW, (DSFL 40k), polyethylene glycol-rhodamine 4.500 MW (PEG-Rh) and fluorescamine (FLCN) were purchased from Molecular Probes, dextran fluorescein isothiocyanate, 12000 MW (dextran FITC 12k) was purchased from Sigma. Dextran sulfate 8000 MW (DS 8k) obtained from ICN Biochemical (OH). Polyethylene glycol 6000 MW and 20000 MW (PEG 6k and PEG 20k, respectively) were obtained from Fluka Chemie AG. Dextran 10000 MW (dextran 10k) was obtained from Pharmacia Fine Chemicals. All other chemicals used were of reagent grade.

2.1. Sendai virus

The Cantell strain of Sendai virus was grown in 10 day old embryonated chicken eggs, and purified from allantoic fluid according to the published method (Al-Ahdal et al., 1986) and suspended in 150 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.4 (NTE). Purified virus (≈ 1 mg/ml) was aliquoted and stored at -70°C until taken for experiments and then were stored at -20°C until used. The virus preparation normally had titers of 5000–10000 hemagglutinating units. Protein concentration of virus was determined by Bio-Rad protein assay and viral lipid concentration was determined by a phosphate assay modified from Allen (1940).

2.2. Virus envelope protein labeling

The labeling of the exterior envelope proteins by fluorescamine (FLCN) was performed by addition from a 5 mg/ml solution in acetonitril (MeCN) to whole virus to give a probe to lysine residue mole ratio of 1.5:1. Viral envelope proteins contain an average of 20 lysine residues per protein (≈ 0.2 mM lys in 1 mg/ml of virus). Standard procedure was as follows: 5 μl of a 5 mg/ml probe solution was added to 0.5 ml of virus (1 mg/ml protein), and incubated in the dark for 2 h at room temperature. Unbound probe was then removed by column chromatography (sephadex G-75). The stock solution of the FLCN in acetonitrile can be stored for up to 12 weeks at 4°C. Labeled virus was stored at 4°C between experiments and on ice during experiments.

2.3. Erythrocyte ghost membranes

Fresh human blood (type A, Rh^+) was obtained from a healthy individual and was washed three times (with a clinical centrifuge) in a rinsing solution (150 mM NaCl, 5 mM NaH_2PO_4, pH 8.0) and the light cells were removed by aspiration. The packed red cells were lysed by a hypotonic solution (5 mM NaH_2PO_4, pH 8.0). The ghosts were resealed in 120 mM KCl, 30 mM NaCl, 10 mM potassium phosphate, pH 7.4 solution (KNP) with 1 mM Mg^{2+}. The newly resealed HEG was suspended in KNP at 1–2 mg/ml, and the protein concentration determined by Bio-Rad protein assay and stored at 4°C at either 1 or 2 mg/ml. Use of older than 2 week HEG was avoided.

2.4. Application of polymer

Since introduction of polymer into the experiment was important, the following two different additions of polymer were employed, pre- or post-incubation, which referred to the cases when the polymer was first introduced into the experiment. Pre-incubation was carried out in a 0.2 ml volume of buffer (150 mM NaCl, 10 mM NaH_2PO_4, pH 7.4 (NPB)) at a specified polymer concentration followed by dilution to the working volume of 2.0 ml with the room temperature NPB containing no polymer. For fusion experiments, the sample was incubated on ice, while, for binding experiments, the incubation was done at each specified experimental temperature. In post-incubation, the initial exposure to the polymer occurs during the dilution step. For post-incubation, the incubated mixture of virus and HEG in a specified temperature NPB was diluted by the room temperature NPB containing the polymer. The incubation time for all experiments except for aggregation experiments was 5 min. In all cases, the virus was added first to the incubation buffer followed by the HEG.

2.5. Anionic polymer (dextran sulfate)

Dextran sulfate 8000 MW or 40000 MW (DS 8k and DS 40k, respectively), was present at various concentrations in NPB for the fusion, binding and aggregation experiments. In the case where UV fluorescence was involved, HEPES was replaced with NaH_2PO_4. When used in fusion experiments, DS was added after incubation of Sendai virus and HEG, which was post-incubation. In binding experiments, both pre- and post-incubation procedures were used, and the aggregation experiments were done with pre-incubation but the incubation time was 30 min in stead of 5 min, as described below.

2.6. Non-ionic polymers

Exposure with the non-ionic polymers; PEG 6k, PEG 20k and dextran 10k, during fusion experiments were both pre- and post-incubation. For the pre-incubation, Sendai virus was added to the polymer solution (on ice) first, followed immediately by HEG, and incubated for 5 min. Then, the incubated sample was diluted with the specified temperature NPB to 2 ml. The procedures for the pre- and post-incubations for virus-HEG binding experiments were as described below.

2.7. Fusion assay

The fusion assay as used is described briefly as follows: Sendai virus was labeled with a 1% solution of R-18 (1 mg/ml) in ethanol by the addition of the probe ($<0.1\%$ EtOH) to the virus, and resulted in a 90% quenching of the fluorescence emission. The fluorescence signal at the maximum emission wavelength in the quenched state is denoted I_o. While fusion proceeds, the increase of fluorescence signal (I) was observed at 585 nm with excitation at 560 nm. Triton X-100 was added to the suspension (0.2% wt/wt) to give the fluorescence signal maximum value (I_t), and was considered 100% dequenching. The extent of fusion is represented by the percent dequenching (%DQ) defined as:

$$\%DQ = \frac{I - I_0}{I_t - I_0} \cdot 100$$

The fusion assay was carried out as follows: 10 μl of R-18 labeled Sendai virus stock solution (≈ 10 μg protein) and 40 μl of HEG stock solution (≈ 40 μg proteins) were mixed with 150 μl of NHB (150 mM NaCl/10 mM Hepes/pH 7.4) and the mixture was incubated on ice for 5 min followed by the virus HEG mixture being suspended in 2 ml of NHB prewarmed at the specified temperature. The Sendai virus to HEG ratio was kept at a 1:4 (wt:wt) ratio in all experiments, except where noted, as a standard for determining fusion ability. The dequenching signal of the fusion events was monitored at various temperatures with a spectrofluorimeter (Perkin–Elmer, LS-5) equipped with a temperature-controlled cell holder with a circulating water bath. Initial intensity of virus with HEG (I_0) was set as 0% fusion and that after addition of Triton X-100 (I_t) as 100% fusion.

2.8. Binding of Sendai virus to human erythrocyte ghosts

R-18 labeled Sendai virus (1mg/ml) was incubated with 40 μl HEG (1mg/ml) in the presence of polymer as in above fusion experiments at various temperatures for 5 min and diluted slowly to 2 ml in room temperature NPB for the pre-incubation case. For post-incubation experiments, NPB containing polymers was used for dilution. The diluted suspension was centrifuged at $4500 \times g$ for 10 min at 4°C. The fluorescence, after dilution to 2 ml, and dequenching by addition of Triton X-100 of both the supernatant and pellet was observed. This procedure was the same for all polymers tested.

2.9. Aggregation assay

Aggregation of particles by the effects of different polymers at various temperatures was monitored by increases in absorbance at 400 nm. All experiments were carried out at the same ionic strength (NPB). Aggregation was determined by subtraction of the absorbance (OD) at λ_{400}; $OD_A = OD_{400}$ (with polymer) $- OD_{400}$ (without polymer). The OD_{400} value after 10 min of equilibration was used and the virus to HEG ratio was $\approx 1{:}4$ (wt:wt protein). The %Δ was defined as the ratio of the above OD_A to the OD_{400} (without polymer) $\times 100$. In every case, samples were allowed 20 min to equilibrate and the ratio (1:4 (wt:wt)) of virus to HEG added was kept a constant. Standard procedure for addition of samples was as follows: Sendai virus (≈ 20 μg) and HEG (≈ 80 μg) were added directly into NPB prewarmed at specified temperature containing specified concentrations of polymer to a final volume of 2 ml. This order of addition was always virus first, immediately followed by the HEG unless otherwise stated. Polymer was present in the reference cuvette at the same concentration as the sample to account for absorbance caused by the

polymer. Sample temperatures were in the range of 4–37°C and were controlled by a circulating water bath.

2.10. Anisotropy

The fluorescent probe, fluorescamine, was used to determine the rotational and/or segmental motion of envelope glycoproteins of whole Sendai virus labeled as above. The probe is inherently non-fluorescent until it binds to primary amines of proteins and degrades quickly in aqueous solution. During anisotropy experiments, 10 μl of virus was used giving a dye concentration in a quartz sample cuvette (2 ml) of 13 nM FLCN, assuming all lysine residues had a dye bound. The concentration of dyes used was lower than that of self-quenching and inner filter effects of the dye (de Bernardo et al., 1974). Steady state emission anisotropy (r) measurements with an excitation wavelength at 381 nm and an emission wavelength at 490 nm were made with the same fluorometer (Parkin–Elmer LS5) as in the fusion experiments, using the following equations:

$$p = \frac{I_{0/0} - I_{0/90} \cdot G}{I_{0/0} + I_{0/90} \cdot G}$$

$$G = \frac{I_{90/0}}{I_{90/90}}$$

$$r = \frac{2P}{3 - P}$$

The polarization, P, is measured and the correction factor G is calculated as shown to result in the anisotropy, $r \cdot I_{0/0}$ and $I_{0/90}$ are the parallel and perpendicular polarized components of the emission fluorescence observed at right angles to an arbitrarily polarized excitation beam and G is the correction factor for the wavelength selector grating of the emission monochrometer.

2.11. Polymer binding to virion

To investigate binding of DS to virions, 40, 80 and 150μg of virus in 0.5 mg/ml DS 40k (10% DSFL 40k) in NPB, were incubated on ice for 30 min at a total volume of 0.5 ml. After dilution of the sample with cold (4°C) DS 40k solution (0.5 mg/ml DS 40k) to 2 ml, they were centrifuged at $23000 \times g$ for 30 min at 4°C. The pellet and supernatant were suspended in an equal volume of NPB and the amount of fluorescence per equal aliquot of the suspension was measured at 520 nm by exciting the fluorescence at 480 nm. The percentage of fluorescent DS bound to the virus to unbound fluorescent DS was determined as follows:

$$\%DS_{bound} = \frac{I_{pellet}}{I_{pellet} + I_{supernatant}} \cdot 100$$

where I_{pellet} and $I_{supernatant}$ are the fluorescent intensities of pellet and supernatant solutions, respectively.

Control experiments with R-18 labeled virus and unlabeled DS 40k were run to confirm that the virus was pelleted under the conditions utilized. Substitution with dextran 10k and dextran FITC 12k or with PEG 6k and PEG-Rh 4.5k was made in similar manner as for the DS binding experiments.

Further investigation of the binding of DS and dextran was made by utilizing the anisotropy of the fluorescein dye bound and unbound. Anisotropy measurements of the fluorescent polymer were obtained from the pellets and supernatants of the above experiments with the fluorescence excitation and emission at the fluorescein maximums, 480 and 520 nm, respectively. The anisotropy of free DSFL 40k or dextran FITC 12k (2.5 μg/ml) in the absence of virus was also determined, as a control for the supernatant samples. Also, binding of DSFL 40k was followed by the change in anisotropy of a solution of 0.5 mg/ml DS 40k (10% DSFL 40k) over time after 36 μg virus was added (by injecting 36 μl of Sendai virus (1 mg/ml)). Binding anisotropy experiments were performed for the samples at two concentrations of DS 40k (0.1 mg/ml DS 40k and 0.5 mg/ml DS 40k) at room temperature.

3. Experimental results

Fig. 1 shows the dequenching signal in the R-18 assay of Sendai virus with HEG in the absence and presence of anionic polymer, DS 8k, as a

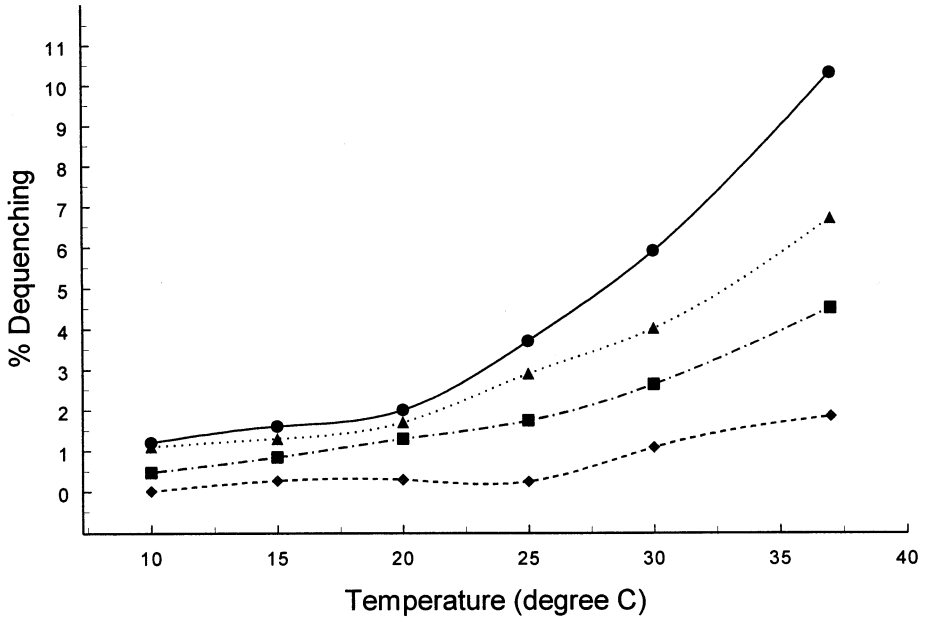

Fig. 1. Fluorescence after 10 min of fusion in the presence of dextran sulfate MW 8000. The protein concentration ratio between Sendai virus and erythrocyte ghost was 1:4 (wt:wt). The ghost and virus were preincubated on ice in 200 μl of buffer (150 mM NaCl; 10 mM HEPES; pH 7.3) for 5 min then diluted to 2 ml in the same buffer with and without containing the anionic polymer which was prewarmed at a specified temperature. The control experiment of Sendai virus and ghosts is without dextran sulfate. Polymer concentrations were; (●) control, (▲) 0.1% DS 8k, (■) 0.2% DS 8k, (◆) 10% DS 8k. Experimental error was within 5%.

function of temperature. The dequenching signal is due to the transfer of the quenched probe from virus to HEG membranes and is considered as the 'extent of fusion'. During the first 10 min, at temperatures below 20°C, fusion was very small as is characteristic of Sendai virus. As temperatures increased above 20°C, increasingly higher extents of fusion resulted until physiologic temperatures were reached. A distinct transition in the extent of fusion curve was seen at 20°C (FuT). When Sendai virus fusion occurred in the presence of DS 8k, the extent of fusion with HEG over time was always less than control values. Fig. 1 shows the extent of fusion with HEG after 10 min with increasing concentration of DS 8k. It is apparent that the extent of the inhibition is dependent on the concentration of DS 8k present. In these experiments, Sendai virus and HEG were allowed to bind during pre-incubation free of any effects of DS 8k followed by dilution with the DS solution. The percent dequenching shown in Fig. 1 exhibits temperature dependence even at concentrations of DS 8k up to 10% (wt/wt). High concentration of DS 8k (10%) reduced the percentage dequenching at temperatures above the FuT even as high as physiological temperatures, however, some dequenching remained.

Some nonionic polymers had noticeable effects on the extent of fusion of Sendai virus (see Fig. 2). The nonionic polymer, PEG 6k and PEG 20k present at 4% (wt/wt), increased the extent of fusion in 10 min while showing no change in the FuT. When another nonionic polymer, dextran 10k, was present at a concentration of 10% (wt/wt), the fusion extent of Sendai virus with HEG at 10 min was slightly lower than control. It should be noted that the effects of PEG and dextran on fusion were due to exposure to the nonionic polymer during pre- and post-incubation.

Aggregation of particles (i.e., HEG, virus, or virus-HEG complexes) caused by the above polymers was examined to explain their effects on fusion. Therefore, aggregation of HEG and virus alone and together were examined for DS 8k (10,

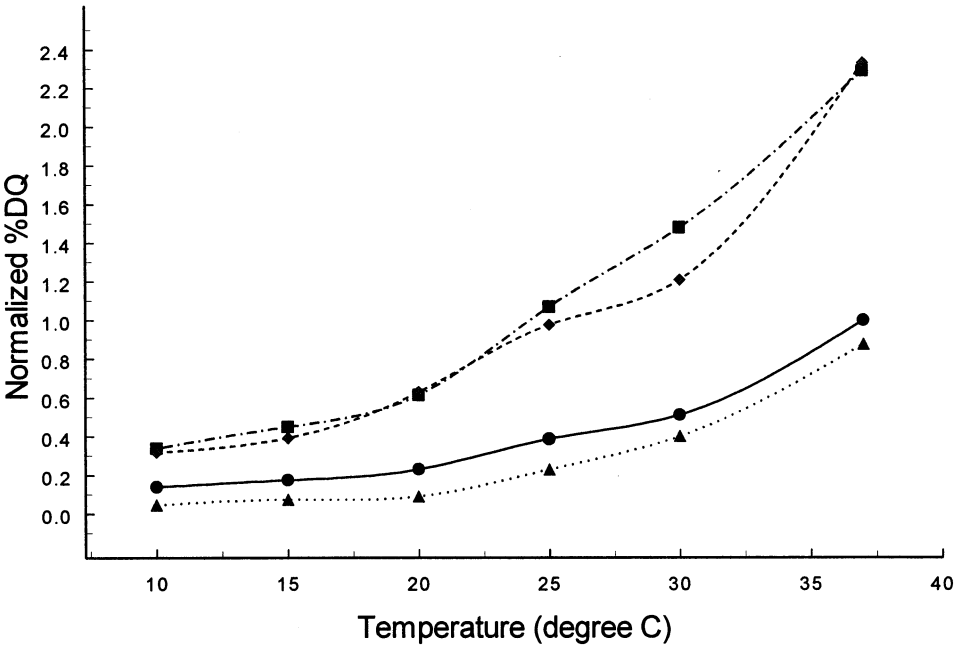

Fig. 2. Normalized fusion of Sendai virus with HEG in a 1:4 (wt:wt) ratio in the presence of nonionic polymers. Polymer solution, where virus and HEG were incubated at a volume of 200 μl for 5 min on ice, was increased to 2 ml with specified temperature buffer containing the same concentration of polymer. Incubation took place on the bottom of the cuvette used in the subsequent fusion experiment. The normalized fusion is expressed as the ratio of dequenching values in the presence of polymer in the buffer solution to the value of the control measured at 37°C (i.e. containing no polymer measured at 37°C). Polymer concentrations were; (●) control (no polymer), (▲) 10% dextran 10k, (■) 4% PEG 6k, (◆) 4% PEG 20k. Experimental error was within 5%.

and 0.1%), dextran 10k (10, and 0.1%) and PEG 6k (4, and 0.1%). The results tabulated in Table 1 showed that the condition with the largest widespread aggregation effect was the 4 and 0.1% PEG 6k solution. 10% Dextran 10k, and 10% DS 8k both aggregated virus maximumly by a doubling of the turbidity at 18°C. PEG and DS, at both concentrations examined, showed detectable changes in aggregation of virus also having maximums at 18°C. Under conditions identical to fusion experiments, the enhancement of fusion seen by PEG is paralleled by an increase in aggregation of virus and HEG. A value above control, having a positive sign (+), is considered to be an increase in aggregation of the particles, while a value with a negative sign (−) is considered as dissociation of the particles. In cases where the order of addition of the particles to the polymer solution were reversed (i.e. HEG first followed by virus), similar aggregation values were also obtained (data are not shown).

The effect of order of exposure of DS 8k to the experimental components (i.e. Sendai virus and HEG) can be seen in the results of virus binding with HEG (see Fig. 3). Binding of Sendai virus to HEG, as the control, showed a temperature dependence with decreased amounts of virus associated with the pelleted HEG at higher temperatures. The presence of 0.1% DS 8k only during the pre-incubation reduced the amount of virus associated with the pelleted HEG by 10–20% over the whole range of temperatures. The effects were enhanced at higher incubation temperatures, however, a rise in the amount of virus associated with pellet at 30°C was possibly due to virus initiating fusion during the pre-incubation. DS 8k added only during post-incubation also had a noticeable effect on the amount of virus associated with pellet even though centrifugation immediately followed the polymer addition. A 10% reduction in the amount of virus associated with the HEG of the pellet for the

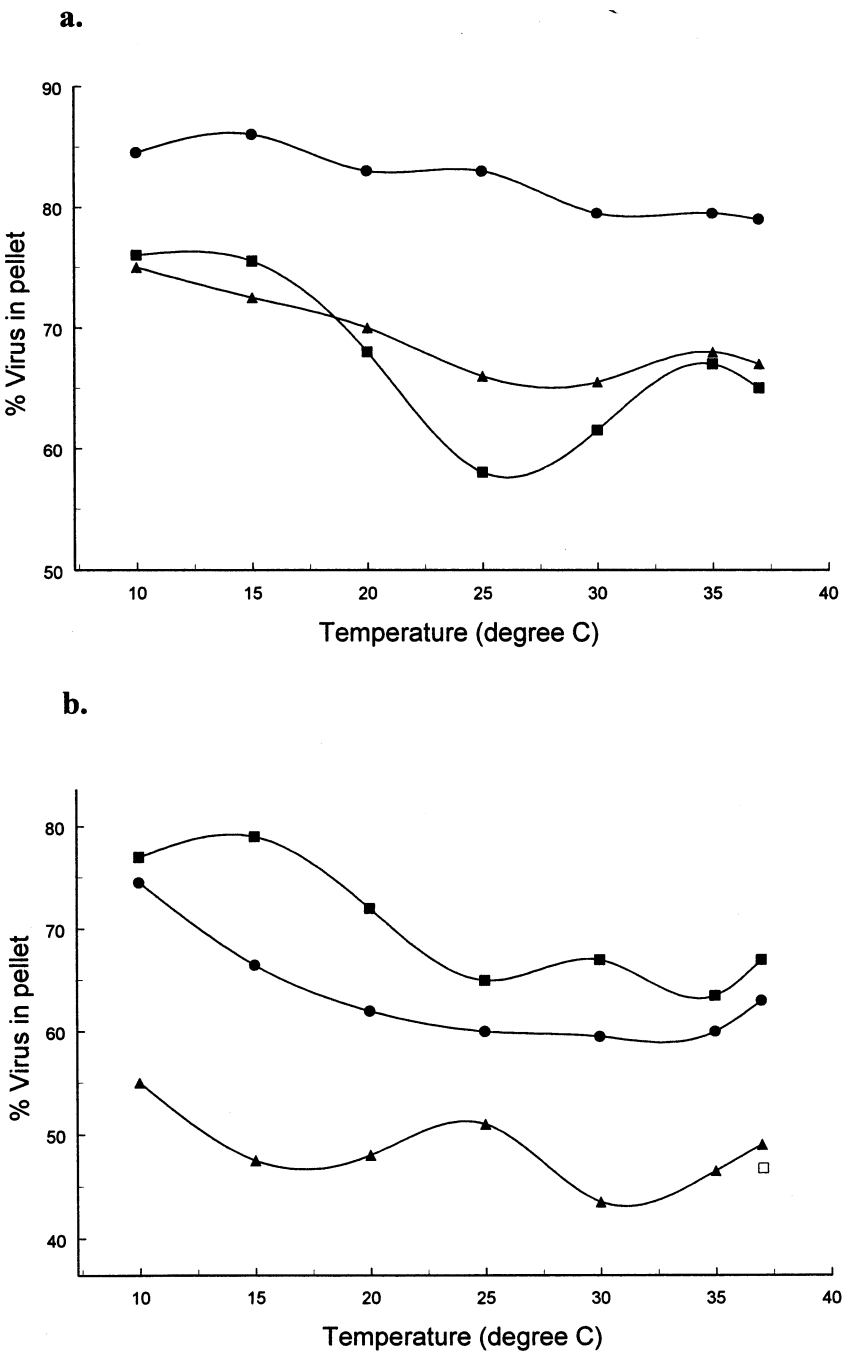

Fig. 3. Sendai virus binding to human erythroctye ghosts at various temperatures. The virus ghost protein ratio was 1:4 (wt:wt) in all experiments and the pre-incubation was in a total volume of 200 μl for 5 min. The concentration of (a) DS 8k was 0.1% (1 mg/ml), (b) dextran 10k was 10 %, and (c) PEG 6k was 4% during the pre (■) and post (▲) incubation. Post incubation consisted of a slow dilution by room temperature NPB to a volume of 2 ml followed by intermediate centrifugation at 4500 × for 10 min. The pre-incubation case ended in a ten times dilution of the polymer concentration before centrifugation, i.e. from 0.1% DS 8k to 0.01% DS 8k. The open box (□) at 37°C represents the value for exposure to dextran by pre- and post-incubation, which shows the reversibility of the pre-incubation effects. Experimental error for each datum point was within 10%.

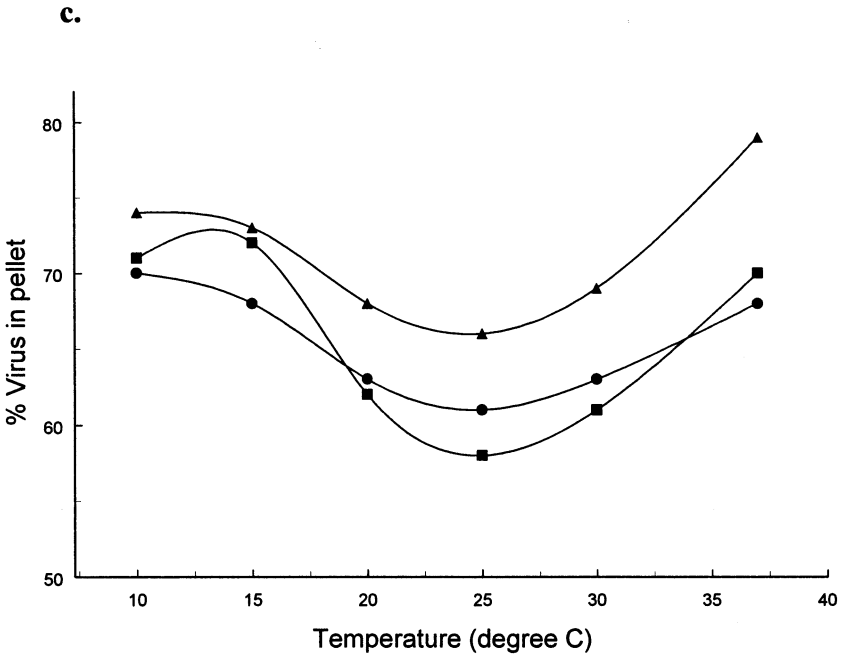

Fig. 3. (*Continued*)

post-incubation case occurred across all temperatures (Fig. 3a).

Dextran also caused dissociation of the virus from HEG when added post-incubation, shown as a 15–20% decrease from control (Fig. 3b). The binding enhancement effect of dextran is seen in the pre-incubation case with the temperature dependence similar to control. To emulate the fusion experiments, dextran was added both pre- and post-incubation at 37°C only and resulted in a large dissociation of virus from the HEG. The experimental results with PEG were different from those of dextran. The pre-incubation with PEG resulted in slightly less binding of virus to HEG than those of the control (no PEG) over the mid-temperature range (20–30°C), while the post-incubation enhanced the binding of virus to HEG than the control over the temperature range examined (see Fig. 3c).

When Sendai virus was incubated with labeled-dextran sulfate 40k (DSFL 40k) the polymer became associated with the virus. The amount of DSFL 40k associated with a 40, 80, and 150 μg virus pellet was determined to be 1.1, 1.4, and 2.8% of the total, respectively, by the ratio of sample fluorescence of the pellet to the total amount of sample (see Table 2a). This resulted in approximately on average 4400 DS 40k molecules per virion by assuming that both DS and DSFL bind to virions at the same ratio.

The binding of DS polymer to Sendai virus was also apparent due to a decrease in mobility of DSFL 40k as shown by fluorescence anisotropy measurements (Table 2a). After binding and centrifugation the Sendai virus associated DSFL 40k of the resuspended virus showed an increased anisotropy of the dye compared to the free polymer in the supernatant (Table 2a). Concentration-dependent binding of DSFL 40k (Table 2b) is shown along with no change in observed anisotropy of the DSFL 40k when the concentration of DSFL 40k present in the bulk is lowered. For both cases, run 1 (0.1 mg/ml DS 40k (10% DSFL 40k)) and run 2 (0.5 mg/ml DS 40k (10% DSFL 40k)), the amount of polymer bound to the virus was assumed to be at the same ratio as polymer (DSFL 40k/DS 40k) in the bulk (i.e. 1/10) and was 1% of the total. The result appeared to be that no interaction occurred between the fluorescein probes since the anisotropy remained the same as for 0.05 mg/ml DSFL 40k shown in Table 2a. DSFL 40k was then allowed to bind to virus over time in the large volume of

Table 1
Degree of aggregation of particles (HEG and/or virus) due to polymer effects

	8°C		18°C		37°C	
	High (%Δ)	Low (%Δ)	High (%Δ)	Low (%Δ)	High (%Δ)	Low (%Δ)
Dextran 10k						
Virus	+0.0 (0.0)	+0.0 (0.0)	+0.005 (100)	+0.004 (88.9)	+0.015 (44)	−0.001 (−33.3)
HEG	+0.003 (1.0)	+0.004 (5.25)	−0.002 (−1.5)	+0.008 (11.3)	+0.005 (3.9)	−0.003 (−4.25)
Virus+HEG	+0.032 (11.5)	+0.001 (1.25)	+0.012 (8.5)	+0.004 (4.7)	+0.004 (2.4)	−0.002 (−3.0)
PEG 6k						
Virus	+0.002 (9.5)	+0.001 (10)	+0.002 (40)	+0.015 (340)	+0.005 (100)	+0.001 (33.3)
HEG	+0.04 (23.4)	−0.008 (−10.5)	+0.074 (66.6)	−0.002 (−2.8)	+0.033 (25)	+0.032 (45.4)
Virus+HEG	+0.048 (28.7)	+0.042 (53.2)	+0.074 (63.2)	+0.042 (49.7)	+0.044 (35.3)	+0.043 (65.4)
DS 8k						
Virus	−0.002 (−3.2)	+0.002 (44.4)	+0.005 (100)	+0.003 (66.6)	+0.0 (0.0)	+0.0 (0.0)
HEG	+0.035 (23.3)	+0.0 (0.0)	+0.016 (12.3)	+0.001 (1.4)	+0.032 (21.6)	−0.006 (−8.5)
Virus+HEG	+0.032 (21.6)	+0.016 (20)	+0.007 (4.8)	+0.004 (4.7)	+0.014 (9.5)	+0.001 (1.5)

Values in the table are the relative absorbance (OD) at λ_{400}; $OD_A = OD_{400}$ (with polymer); OD_{400} (without polymer).
The OD_{400} value after 10 min of equilibration was used and the virus to HEG ratio was $\approx$1:4 (wt:wt protein).
The %Δ was defined as the ratio of the above OD_A with the OD_{400} (without polymer) × 100.
Polymer concentrations in standard buffer (150 mM NaCl; 10 mM NaH_2PO_4; pH 7.4) for the high and low compound concentrations were: PEG 6k (4/0.1%); Dextran 10k (10/0.1%); and DS 8k (10/0.1%).
The S.D. for all OD values was $\pm$0.002.

a cuvette (2 ml) (Table 2c). Results in Table 2c shows that for the binding of a low concentration of 0.05 mg/ml DSFL 40k without any unlabeled polymer present the anisotropy rose until it reached a maximum after 30 min. The anisotropy value is representative of the free and bound DSFL 40k and at the maximum value at 30 min does not reach the value of DSFL 40k bound to the pelleted virus. However, it should be noted that for the much larger volume a time-dependent (diffusion limited) binding can be seen.

Investigation of binding of dextran and PEG to Sendai virus with the same procedure as DS but using dextran-FITC 12k and PEG-Rh 4.5k, showed that dextran and PEG did not bind to Sendai virus. Dextran-FITC 12k and PEG-Rh 4.5k were detected in the pelleted virus less than 0.5% of total polymer after 30 min of incubation at various virus concentrations used for the DS experiments. Also, the probe attached to the dextran that was detected with the resuspended pellet exhibited an anisotropy identical to that of free polymer (data are not shown).

Binding of DS to Sendai virus was shown to have a direct effect on the mobility of viral envelope glycoproteins as determined by anisotropy of the fluorescent probe bound to the proteins. In addition to the experiment using DSFL 40k mentioned above, anisotropy measurements of fluorescamine labeled envelope glycoproteins of whole virus were made (Fig. 4). A pronounced decrease in the anisotropy occurred when temperatures above 20°C were reached, but this change was inhibited by the presence of 0.5 mg/ml DS 8k.

Both nonionic polymers altered the anisotropy under the same temperature conditions as DS. The concentrations of PEG 6k and dextran 10k were the same as used in the fusion experiments. PEG and dextran, having very different molecular structures, produced different results for virus envelope protein anisotropy. PEG had effects similar to that shown for 0.5 mg/ml DS 8k where the increased anisotropy was pronounced above 20°C. Dextran produced results of an intermediate value for the anisotropy across the temperature range, decreasing slightly at temperatures above 20°C. DS resulted in an inhibition of fusion and an increase in anisotropy while dextran also inhibited fusion but lowered the anisotropy

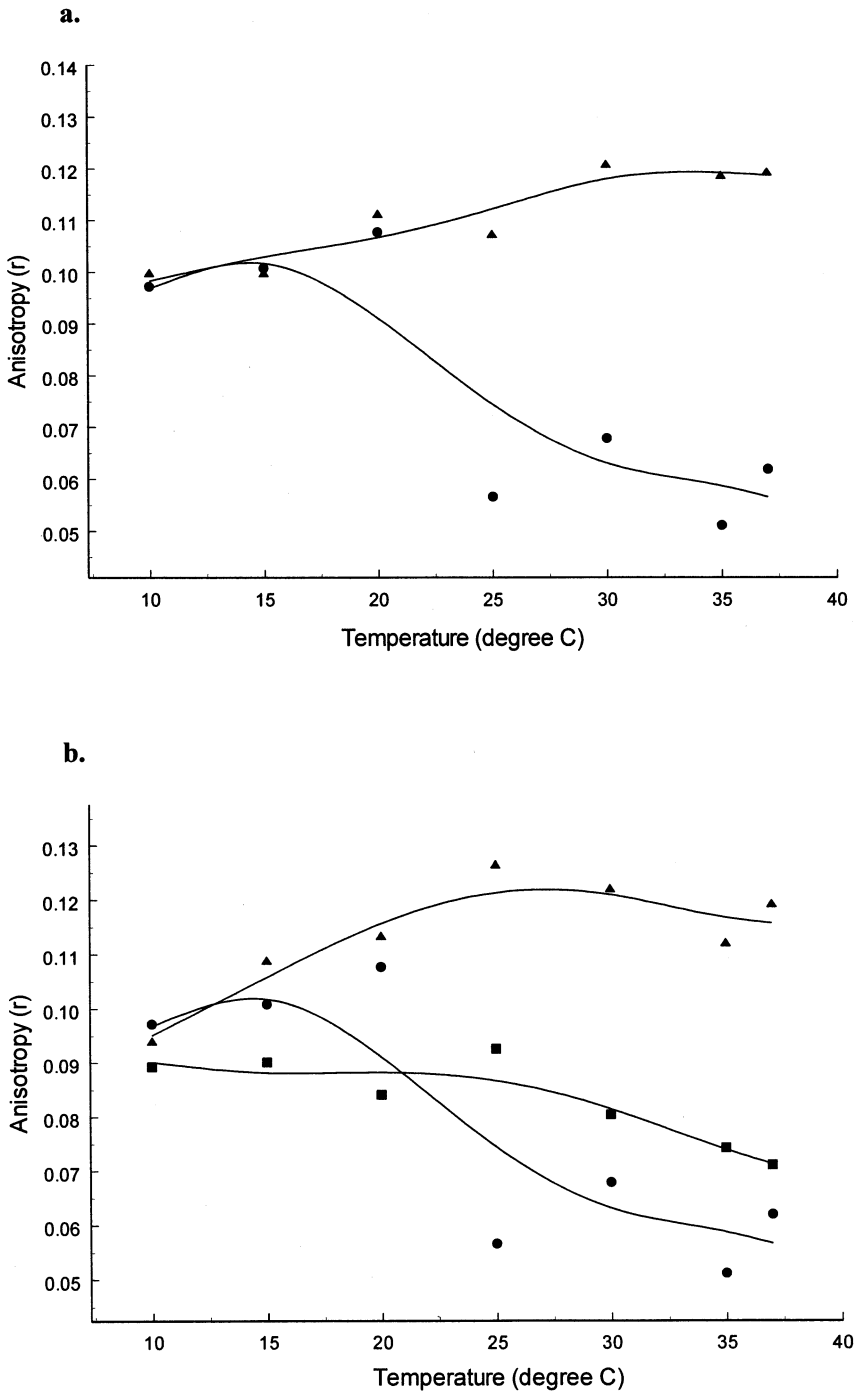

Fig. 4. Anisotropy of Sendai virus envelope glycoproteins labeled with fluorescamine. (a) To allow binding of DS, 50 μg of Sendai virus labeled with fluorescamine was incubated for 30 min in 2 ml of 0.5 mg/ml DS 8k. Closed circles (●) represent probe anisotropy free of polymer while closed triangles (▲) represent probes of virus with prebound dextran sulfate 8k. (b) The same conditions as for DS were used for the nonionic polymers 4% PEG 6k (▲) and 10% dextran 10k (■). The samples were equilibriated for 20 min at each temperature before the anisotropy was obtained. Experimental error was within 15%.

Table 2
Percent of bound and unbound DSFL (dextran sulfate-fluorescein) to Sendai virion and their fluorescence anisotrophy

a)

Amount of virus (μg)		% of total DSFL 40k	Anisotropy r (a:)
40	Pellet	1.1	0.1131
	Supernatant	98.9	0.0429
80	Pellet	1.4	0.1058
	Supernatant	98.6	0.0430
150	Pellet	2.8	0.1048
	Supernatant	97.2	0.0437

b)

		run 1	run 2
	Supernatant	0.045	0.0462
	Pellet	0.1058	0.1090

(c)

Time (min)	
0	0.0429
5	0.0467
30	0.0933

(a) Amount of DSFL 40k bound to Sendai virus. Sendai virus was incubated at a volume of 0.25 ml with 0.5 mg/ml DS 40k (10% DSFL 40k) for 30 min on ice followed by dilution to 2 ml with the same solution and immediately centrifuged at $23\,000 \times g$ for 30 min.
The average number of DS 40k polymers per virion was calculated to be 4400/virion with use of equation, the experimental data of DS binding, concentration of the total fluorescence probe, and the total lipids of virus in the experiments, assuming the virus is a spherical shell of lipid bilayer of the diameter 0.2 μ.
In all cases the anisotropy of the virus pellet associated DSFL 40k was similar to the results in the tables above.
(b) Binding of DSFL 40k to Sendai virus: 150 μg of virus incubated at room temperature in run 1 with a total concentration of 0.5 mg/ml DS 40k (10% DSFL 40k) and run 2 in a total concentration of 0.1 mg/ml DS 40k (10% DSFL 40k).
After centrifugation the pellet was resuspended in the same concentration of DS 40k without DSFL 40k.
(c) Binding of dextran sulfate 40k to Sendai virus over time as determined by Anisotropy.
Sendai virus (≈ 40 μg) was injected into a solution of 0.05 mg/ml DSFL 40k at 20°C and the anisotropy of the labeled polymer was followed over time.
Time zero represents free polymer before addition of virus was made.

below the FuT and increased it above the FuT. PEG, the other nonionic polymer, produced enhanced fusion and increased anisotropy. This indicates a possible difference in the interaction of the two nonionic polymers with the Sendai virus envelope glycoproteins.

4. Discussion

One method of attachment of Sendai virus to HEG membrane proteins is mediated by a specific receptor–ligand interaction between the HN protein of the virus and sialic residues of the HEG. By way of these attachments, the virus envelope approaches to the target cell. The viral HN protein is thought to extend as far as 12 nm from the viral envelope limiting this distance of approach for the opposing membranes, assuming the target binding sites do not extend any significant distance. The anionic polymers, dextran sulfate, heparin and chondrotin sulfate, have been reported to inhibit fusion of Sendai virus with HEG (Ohki et al., 1992; Zschörnig et al., 1993). The same concentration dependence of dextran sulfate inhibition was shown here (Fig. 1) as those obtained by Ohki et al. (1992). Although fusion was inhibited, it was found that there was no apparent change in FuT. As defined above, FuT is where fusion of virus to target cells

(e.g. HEG) starts to increase sharply with temperature.

The enhancement of fusion of Sendai virus by nonionic polymers such as PEG is well known (Hoekstra et al., 1989; Arnold et al., 1991) and is believed to be due to stabilization of the association of virion to the target membrane. Hoekstra et al. (1989) showed that the presence of PEG increased only the rate of fusion of the virus with HEG without changing the final extent of fusion. The proposed dehydrating effect of PEG must affect the amount of surface contact as its method for the enhanced binding and increase in fusion rate since the distance between the apposed membranes was unaffected (Wagner and Ohki, 1998) in the presence and absence of PEG. The Sendai virus used here exhibited the same fusion characteristics in the presence of PEG as that of other authors (Hoekstra et al., 1989); an enhanced extent of fusion as well as an unaltered FuT, which has been denoted above as the fusion threshold temperature. From the aggregation results shown in this study, the effects of PEG do not seem to be particle specific but instead depends on its concentration since the higher concentration (e.g. 4%), increased aggregation for Sendai virus alone, HEG alone, and Sendai virus with HEG. Exclusion of PEG from membrane surfaces may provide the driving force necessary for HEG aggregation (depletion flocculation) (Arnold et al., 1990).

Dextran, the other nonionic polymer, has been shown to aggregate red cells with a decrease in this effect as the molecular weight decreases (Gallez and Coakley 1986; Coakley et al., 1991). Coakley et al. (1991) reported that molecular weights as low as 20k for dextran did not cause aggregation of red cells unless the cells were pretreated with pronase. Dextran 10k did not aggregate red cells even with pronase treatment, even at concentrations as high as 8% wt/vol dextran. HEG was aggregated poorly under the conditions used here, which exceeded 8% and agree with the results stated above of Coakley et al. (1991). In the presence of 10% dextran 10k a doubling of the aggregation of Sendai virus at 18°C resulted, but dropped off to 44% at 37°C. The case of Sendai virus with HEG was opposite to the virus alone case where the two particles have a receptor–ligand interaction. The receptor-mediated binding of Sendai virus with HEG, in the presence of 10% dextran 10k, appears to be enhanced by increases of 11.5, 8.5, and 2.4% at 8, 18, and 37°C, respectively, as determined by aggregation results (Table 1). At higher temperatures the dextran-induced self-aggregation of Sendai virus may be what contributes to the decrease in the aggregation of Sendai virus with HEG (Table 1). This helps to explain the decreased extent of fusion shown in the presence of 10% dextran 10k.

Inhibitory effects of dextran sulfate are most likely made up of multiple mechanisms, including the inhibition of the initial binding step, increased dissociation of bound virus, etc. Hoekstra and Klappe (1986) showed that detachment ($\approx$ 10–20%) of virus from HEG occurs when prebound virus is diluted with warm buffer (Hoekstra and Klappe, 1986). During the Sendai virus and HEG binding experiment the presence of DS 8K in the dilution buffer (post-incubation) is believed to inhibit the rebinding of dissociated virus. Also, the inhibition effect may be due to the increased dilution volume which may reduce the chances of the virus not coming in contact with HEG. It is possible that the inhibiting effects of DS 8K act also by binding to the HN protein thus hindering bound virus from finding a suitable fusion site. This inhibition would be due to competition for HN protein binding sites resulting in dissociation of the virus. If DS binds to the membrane bound virus then the virus may become stationary and unable to change its initial position which may or may not be suitable for fusion. If this is the case and the virion is unable to change its position, once a higher temperature is reached, the neuraminadase activity may cause the dissociation of the virion from the target membrane due to digestion of the binding moiety. This may have been the cause for the lessened binding in Fig. 3 when DS 8k was present during the pre-incubation.

Inhibition of binding of Sendai virus to target membranes by steric factors is not hard to imagine since the binding of 4400 molecules to a single virion, in the presence of 0.1% DS in the virus suspension, could present problems for the virus during its approach to a target membrane. Due to its multiple negative charges per monomer unit as well as having a branched polymer structure, DS should be predicted to have an extended conforma-

tion in aqueous solution (Arnold et al., 1990). If DS 8k polymer lies down on the surface of the virion when it binds, the number bound on average would cover ≈ 67650 nm^2 (54% surface coverage) of an average virion of diameter ≈ 200 nm. Therefore, the less surface area available for binding, the harder it may be for the virion to bind to the target cells which results in less aggregation, and thus, in turn, would result in smaller extent of fusion.

The binding of DS 8k to virions seemed to restrain the viral envelope glycoproteins mobility above 20°C (see Fig. 4a & b), while Lee et al. (1983) showed that viral protein mobility is necessary for fusion. Lee and coworker also showed in the same work that binding of free glycophorin caused a decrease in mobility of the viral envelope proteins at 37°C. If the increased mobility of Sendai virus envelope glycoproteins is necessary for the fusion event, inhibition of this increase would seem to inhibit fusion. The finding, that the envelope glycoprotein mobility increase occurs just before the FuT, may possibly reflect a rearrangement of the proteins on the surface of the virus from an aggregated state. However, it was found by Ohki (unpublished data) that when virus was bound to HEG, viral glycoprotein mobility was restricted more than the control above FuT. This suggests that the segmental mobility of viral glycoproteins may not be necessary related to the trigger for protein-induced viral membrane fusion.

The nonionic polymers, PEG 6k and dextran 10k, have very different molecular structures that may account for their different effects on fusion, aggregation, and probe anisotropy. PEG has ethylene glycol, a polar molecule, as a monomer and is a linear polymer while dextran has glucose as its monomer, primarily linked $(1\rightarrow6)$-α-D-glucopyranosyl residues with some $(1\rightarrow3)$ linkages as branches, and is considered a ramified (branched) polymer. PEG seems not to have a direct physical interaction with the Sendai virus surface but influences the fusion and aggregation via a volume exclusion property. The anisotropy change induced by PEG may have its origin in this same property. Dextran and PEG did not bind to Sendai virus under conditions while DS did. These results indicate the charges of DS may play an important role in binding. This may also indicate the importance of charge groups on gangliosides in the binding process. Although sugar moieties of some glycoproteins (Hayman et al., 1973; Kumar and Sarker, 1996) do have affinity for Sendai virus glycoproteins, dextran does not. The effects of dextran on Sendai virus must then be indirect possibly by volume exclusion or a change in dielectric constant of the virus surface. A weak adsorption of the dextran polymer is not ruled out by the binding experiments and could result in depletion flocculation if the concentration of the adsorbed polymer is higher than the bulk (Van Oss et al., 1990). This type of aggregation could account for the results seen for dextran with virus in Table 1.

Complete inhibition of Sendai virus fusion by DS may be possible. Ohki et al. (1992) reported from electrophoretic mobility studies that DS 40k binding to Sendai virus saturated at 10 mg/ml (1.0% DS 40k). It was seen here that fluorescence dequenching of Sendai virus still occured at 100 mg/ml DS 8k (10%) and a surface coverage of 54% was estimated even at 0.5 mg/ml DS 40k, only 0.05%. Under an expected saturation binding condition for DS 8k, dequenching is still seen. However, transfer of the R-18 probe from the viral membrane to target cell without fusion can result in about 3–4% dequenching when measured over the initial 10 min period (Ohki et al., 1998b).

Lastly, a point about the binding of Sendai virus, as shown in Fig. 3, may be made in regards to FuT. There is some indication here that the FuT may actually be higher (Lee et al., 1983Ohki, unpublished data) than that currently thought (i.e. 18–20°C) (Hoekstra et al., 1985, 1989; Aroeti et al., 1990). The decrease in the pellet associated virus starts to reverse itself as the temperature approaches 30°C and this temperature correlates well with that predicted by Lee et al., (1983) by rotational mobility studies of viral envelope proteins. The result of pre-incubated Sendai virus and HEG from Fig. 3 along with the fusion results from Fig. 1 show a possible FuT of ≈ 25°C to be a closer prediction.

The inhibition and enhancement of fusion of Sendai virus by application of the above mentioned polymers appeared to occur without affecting the characteristic FuT. This leads us to believe that the fusion process which was occurring was essentially the same as that of the natural virus. Also, even

though an anionic polymer attached to the viral proteins, hindering their motions at higher temperature, binding and fusion still occurs. This is an indication that viral envelope protein mobility is not the trigger for protein-induced viral membrane fusion. From our recent studies using Sendai virosomes (reconstituted Sendai envelope which bears the envelope glycoproteins, HN and F, but does not contain the viral internal proteins), we think that the temperature characteristics of Sendai viral fusion (especially FuT) seems to be controlled by the viral internal proteins (Ohki et al., 1998a, in preparation).

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