Lateral Mobility of Both Envelope Proteins (F and HN) of Sendai Virus in the Cell Membrane Is Essential for Cell-Cell Fusion*

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Fluorescence photobleaching recovery was employed to study the effects of specific immobilization of Sendai virus envelope glycoproteins (F, the fusion protein, and HN, the hemagglutinin-neuraminidase) on the virally mediated fusion of human erythrocytes. Lateral immobilization of varying fractions of F and/or HN (after virus adsorption and hemaggulination, but before fusion) was achieved by cross-linking them with succinyl concanavalin A (inhibiting both F and HN) or with specific rabbit IgG directed against either F or HN. Alternatively, agglutinated cells were treated with low concentrations of the above proteins (inducing only minor inhibition of either mobility or fusion), and immobilization of F and/or HN was induced by cross-linking with a secondary antibody; this protocol ensured a minimal contribution of direct binding to the viral proteins to the inhibition of fusion. Our results demonstrate that lateral immobilization of either F or HN results in a strong inhibition of cell-cell fusion and a much weaker inhibition of virus-cell fusion. The level of cell-cell fusion was directly correlated with the level of laterally mobile viral glycoproteins in the cell membrane (either F or HN). We conclude that lateral mobility of both F and HN in the red cell membrane is essential for cell-cell fusion and that not only F but also HN has a role in this fusion event. The possible reasons for the different dependence of cell-cell and virus-cell fusion on viral glycoprotein mobility are discussed.

Enveloped viruses penetrate animal cells by a fusion event, which takes place between the viral envelope and the cellular plasma membrane, or between the virion and the lysosomal membrane following adsorptive endocytosis (1). The fusogenic activities of enveloped viruses have been identified with specific viral envelope glycoproteins (1–5), making virally mediated fusion the best characterized system of biological membrane fusion and therefore an attractive one for studies on the fusion mechanism.

Paramyxoviruses fuse directly with the cellular plasma membrane at neutral pH values. Among this family, the fusogenic activities of Sendai virus were investigated most extensively. The envelope of Sendai virions contains two glycoproteins: the fusion protein (F),1 which is thought to mediate membrane fusion, and the hemagglutinin-neuraminidase (HN) protein, which mediates virus binding to cellular receptors and cell agglutination (2, 6). However, recent findings raised the possibility that the HN protein may also play an active role in the fusion event (7–11).

The mechanism by which viral envelope proteins induce fusion is still unknown. Using fluorescence photobleaching recovery (FPR), we have recently demonstrated that the envelope proteins of Sendai virions (F and HN) become laterally mobile in the membrane of human erythrocytes following fusion (12). Moreover, utilizing the fact that viral envelope-cell and cell-cell fusion are separable events (13, 14), we found that conditions that selectively inhibit cell-cell fusion but not virus-cell fusion also lead to a reduction in the level of laterally mobile viral glycoproteins in the target cell membrane (15, 16). These findings demonstrated that lateral immobilization on the viral glycoproteins in the target cell membrane is not an immediate consequence of viral envelope-cell fusion and suggested that it may have an essential role in virally mediated cell fusion.

In order to establish that lateral motion of the viral glycoproteins (either both or only one glycoprotein species) in the target cell membrane is an essential step in the fusion mechanism, it was necessary to demonstrate that specific immobilization of the viral glycoproteins results in a concomitant inhibition of virally mediated cell fusion. In the present work, we investigated this question by measuring the lateral mobility of F and HN in preparations where the viral glycoproteins were cross-linked (together or separately) by succinyl concanavalin A (SConA) or by specific antibodies. The findings show that lateral immobilization of either F or HN (after the stages of virus binding and hemaggulination, but prior to fusion) largely inhibits cell-cell fusion, while the inhibitory effect on viral envelope-cell fusion is significantly weaker. These results strongly suggest that lateral mobility of the viral glycoproteins in the erythrocyte membrane is an essential step in the induction of cell fusion by Sendai virions and indicate that both F and HN play a role in this process.

MATERIALS AND METHODS

Reagents—Octadecylrhodamine B chloride (OAR) and tetramethylrhodamine (TMR)-5,6-isothiocyanate were purchased from Molecular Probes (Junction City, OR). Bovine serum albumin, ConA (grade III), peroxidase-coupled ConA, and rabbit antiserum directed against

1 The abbreviations used are: F, Sendai virus F protein; HN, Sendai virus hemagglutinin-neuraminidase protein; FPR, fluorescence photobleaching recovery; ConA, concanavalin A; SConA, succinyl concanavalin A; OAR, octadecylrhodamine B chloride; TMR, tetramethylrhodamine; GAR IgG, goat IgG directed against rabbit IgG; HAU, hemagglutinating units; D, lateral diffusion coefficient; Rm, mobile fraction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
ConA were from Sigma. SConA was prepared according to Gunther et al. (17). Affinity-purified goat IgG directed against rabbit IgG (GAR IgG) was a generous gift from Dr. Benni Geiger (Immunological Chemistry, Weizmann Institute of Science, Rehovot, Israel).

Viruses—Sendai virus (Z strain) was grown in the allantoic sac of 10-11-day-old embryonated chicken eggs, harvested 48 h after infection and purified as described earlier (15, 16, 19). The virus was resuspended in 160 mM NaCl, 20 mM Tricine, pH 7.4 (solution A), and stored at 70 °C. Viral protein concentration was determined by a modified Lowry procedure (20). Viral hemagglutinating activity (around 13,000 HAU/mg of viral protein) was measured in hemagglutinating units (HAU) as described (18).

Antibodies and Fab' Fragments Against Viral Envelope Proteins—The antibodies employed in the present studies were described by us earlier (12, 16, 21). They were raised in rabbits by intracutaneous injections of vesicles containing either F or HN proteins (12, 21). Monovalent Fab' fragments labeled with TMR-isothiocyanate (TMR-Fab') were prepared from the IgG fractions as described (12, 15). They did not cross-react with erythrocyte membrane components, as demonstrated by their failure to bind to erythrocytes which were not exposed to Sendai virions (12). Moreover, no cross-reactivity between the anti-F and anti-HN antibodies was detected by SDS-PAGE of whole Sendai virions followed by immunoblotting (12, 21).

Interaction of Sendai Virions with Human Erythrocytes—Fresh human blood (group O, Rh-positive) was obtained from a blood bank and stored with sodium citrate up to 7 days at 4 °C. Prior to use, erythrocytes were washed with solution A, diluted to 2% (v/v), and incubated with the same buffer (4 °C, 15 min, in a total volume of 1 ml) with 400 HAU/ml of Sendai virions. The virions were earlier subjected to mild sonication and filtration through a 0.45-μm Acrodisc filter (Gelman, Ann Arbor, MI) to eliminate large viral aggregates (12). After washing twice with cold solution A, the agglutinated cells were suspended in 0.25 ml of the same solution equilibrated to 19 °C, as described (18). The cells were washed twice with cold solution A and incubated for 30 min at 37 °C to induce fusion. In cases when a second antibody (against ConA or against rabbit IgG) was required, it was performed prior to fusion at 19 °C, exactly as described for the first incubation. The degree of cell-cell fusion was evaluated by determining the percentage of fused cells among the total cell population, employing phase contrast microscopy (12, 19).

Determination of Virus-Cell Fusion—Viral envelope-cell fusion was determined by Rₐ fluorescence dequenching (23), as described by us earlier (15, 16). Rₐ was incorporated into the envelope of native Sendai virions (15, 16, 23) to yield 2-3 mol% Rₐ in the viral envelope, with a quenching degree (Q') of 60-80% (Q' = ([Fₐ - F] / 100)/Fₐ), where Fₐ and F are the fluorescence intensities prior to and after addition of 1% v/v Triton X-100. The residual quenching (RQ) after incubation of the virions with the cells was determined similarly, from the fluorescence ratio before and after the addition of Triton X-100. The percentage of fused virions was determined from the dequenching (DQ) of the fluorescence during the incubation with the cells, according to the formula \( DQ = 100(Q' - RQ)/Q' \). The fluorescence was measured with a Perkin-Elmer MFP-4 spectrofluorometer (\( \lambda_{ex} = 560 \text{ nm}; \lambda_{om} = 590 \text{ nm} \)).

Rₐ-labeled virions were incubated with intact erythrocytes as described above. After fusion, hemoglobin was removed by washing twice with solution A after lysis. The wash after fusion removes the viral particles that detach from the cells during the incubation period (24). Thus, this procedure measures the percentage of virions fused with the cells out of the total amount of cell-associated virions (adsorbed or fused) after the completion of the fusion-promoting incubation.

Determination of the Number of Fused Virions per Cell—The average number of virions fused with each cell was determined by multiplying the fraction of fused virions (measured as described above) by the total number of cell-associated virions after incubation at 37 °C (16). The latter parameter was determined by subcellular fractionation of the washed ghosts obtained after fusion of the cells with Rₐ-labeled virions using 1% (v/v) Triton X-100 and measuring the fluorescence intensity. The level of Rₐ fluorescence per μg of viral protein (derived from the fluorescence of a known amount of virions solubilized with Triton X-100 and used for calibration (24, 25)) was assumed to contain 1.3 × 10⁶ viral particles (24).

Fluorescence Photobleaching Recovery—Lateral diffusion coefficients (D) and mobile fractions (Rₐ) of the viral glycoproteins in the cell membrane were measured by FPR (26, 27) using a previously described apparatus (28). The bleaching conditions in the FPR studies were shown not to alter the lateral mobilities measured (29, 30). Following incubation with the virions as described above (under identical conditions with human erythrocytes), the ghosts obtained by the viral hemolytic activity were treated with glass coverslips precoated with polylysine (10-min incubation of the coverslip with 5 μg/ml poly-L-lysine in 20 mM phosphate buffer, pH 8). The cells were labeled with anti-F or anti-HN TMR-Fab' fragments (100 μg/ml, 30 min, 22 °C, in solution A containing 0.2% bovine serum albumin), washed twice, and wet-mounted in the same solution for the FPR experiments, performed at 22 °C.

The monitoring laser beam (529.5 nm, 1 microwatt, argon ion laser) was focused through the microscope to a Gaussian radius of 0.30 μm with a 100× oil immersion objective. A brief pulse (6 milliwatt, 20 ms) bleached 60-70% of the fluorescence in the illuminated region. The time course of fluorescence recovery was followed by the attenuated monitoring beam. D and Rₐ were extracted from the fluorescence recovery curves by nonlinear regression analysis (31). Incomplete recovery was interpreted to represent fluorophores which are immobile on the experimental time scale (D < 5 × 10⁻⁸ cm²/s).

RESULTS

Effects of SConA on Sendai Virus-mediated Fusion and on Viral Glycoprotein Mobility—The envelope proteins of Sendai virions contain mannose residues (32) and are therefore potential binding sites for the tetrameric lectin ConA of its dimeric derivative, SConA. SDS-PAGE of whole Sendai virions followed by electrophoretic and labeling with peroxidase-conjugated ConA showed that the lectin binds to both F and HN (data not shown). Thus, the above lectins provide a simple and straightforward means to cross-link the viral glycoproteins and investigate the effects of the cross-linking on viral glycoproteins mobility and on the virally mediated fusogenic activities.

Human erythrocytes were incubated with SConA after being agglutinated by Sendai virions (to avoid any interference of the lectin with the agglutination step), but prior to fusion (see "Materials and Methods"). Fig. 1 demonstrates that SConA inhibits both virus-cell and cell-cell fusion and that the inhibition of the latter process is significantly more pronounced. Preincubation of erythrocytes with 20 μg/ml ConA was insufficient to inhibit the fusion of Sendai virions with human erythrocytes (data not shown).

![Fig. 1. Effect of SConA on virus-cell and cell-cell fusion.](image-url)
SConA (the highest lectin concentration employed) prior to incubation with the virions did not inhibit any of the fusogenic activities, demonstrating that the inhibitory effects of SConA are not mediated through binding to cellular components. A similar preincubation with 20 μg/ml ConA produced a mild inhibition (15–20% of virus-cell and cell-cell fusion; for this reason, SConA was employed in all further studies rather than ConA. These findings are in accord with an earlier report (33) that preincubation of erythrocytes with ConA produced only a weak inhibition of Sendai virus-mediated hemolysis.

At least part of the inhibition of cell-cell fusion by SConA may be the direct result of the reduction in virus-cell fusion. The "expected inhibition" of cell-cell fusion due to the lower number of virions fused per cell can be calculated for each SConA concentration, using a calibration curve which correlates the level of cell-cell fusion with the number of fused virions per cell. The calibration curve (Fig. 2) was constructed by incubating erythrocytes with decreasing virus concentrations, resulting in the fusion of fewer virions per cell and in lower cell-cell fusion. Since the reduction in virus-cell fusion in the presence of each SConA concentration is known (Fig. 1), the calibration curve yields directly the contribution of this reduction to the inhibition of cell-cell fusion (the expected inhibition). Comparison of the expected inhibition curve with the actual decrease in cell-cell fusion (Fig. 1) clearly demonstrates that most of the inhibitory effect of SConA on cell-cell fusion is not the result of the mild reduction in virus-cell fusion, and that the sensitivity of cell-cell fusion to inhibition by SConA is much greater than that of virus-cell fusion.

In order to examine the effects of cross-linking the viral glycoproteins with SConA on their lateral mobility, erythrocytes were treated with SConA exactly as in Fig. 1. After fusion at 37°C, the viral glycoproteins (F or HN) were labeled by specific monovalent TMR-Fab' fragments, and FPR experiments were conducted to determine the lateral mobility of F and HN in the target cell membrane. The results of these experiments (Fig. 3) demonstrate a strong reduction in R₂ of both F and HN in the target cell membrane upon elevation of the SConA concentration, accompanied by a decrease in the percentage of cells on which laterally mobile viral glycoproteins could be detected. Cells which displayed mobile fractions of F or HN which were high enough to enable determination of their D values showed the same diffusion coefficients as in the absence of lectin (3.1–3.3 X 10⁻⁶ cm²/s), indicating that the viral envelope proteins whose motion is inhibited by SConA become completely immobile on the FPR experimental time scale.

Part of the inhibition of the virally mediated cell-cell fusion by SConA could result directly from the binding of the lectin to F and/or HN, rather than from their immobilization. To achieve an effect which is mostly due to cross-linking of the viral glycoproteins, erythrocytes agglutinated with Sendai virions were incubated with a low (1.5 μg/ml) concentration of SConA, which by itself induces a minor effect on fusion or on viral glycoprotein mobility (Figs. 1 and 3). This was followed by further cross-linking of the SConA by a 1:20 dilution of rabbit antiserum directed against ConA (details of the protocol are given under "Materials and Methods"). After fusion at 37°C, the F or HN proteins were labeled with TMR-Fab' fragments, and the cells were taken for FPR measurements. The results (Table I) demonstrate a concomitant effect of the combination of a low SConA concentration with anti-ConA on R₂ of the two viral glycoproteins and on cell-cell fusion. The further inhibition of the viral fusogenic activities by the anti-ConA serum (which is the major part of the inhibition, since the low SConA concentration without the antiserum had a very weak effect) is solely due to cross-linking of the SConA molecules which are bound to the viral glycoproteins; this is exemplified by the finding that incubation of virus-agglutinated cells which were not exposed to SConA with the same dilution (1:20) of the antiserum did not affect either cell-cell or virus-cell fusion. Once more, cell-cell fusion was much more sensitive than virus-cell fusion to
Effects of SConA further cross-linked by anti-ConA on the lateral mobility of the viral glycoproteins and on virally mediated fusion.

Erythrocytes agglutinated by Sendai virions were incubated with 1.5 μg/ml SConA as described under "Materials and Methods." After washing, samples designated SConA + anti-ConA were further incubated with a 1:20 dilution of anti-ConA rabbit antiserum and warmed to 37°C to induce fusion (see "Materials and Methods"). In cases where anti-ConA and/or SConA were omitted, they were replaced by the incubation buffer. After labeling with anti-F or anti-HN TMR-Fab' fragments, the mobile fractions (Rf) and diffusion coefficients (D) were measured by FPR on 50-60 cells in each case, as in Fig. 3. The number of fused virions per cell (FV) and the percentage of fused cells were determined in four separate experiments in each case as described under "Materials and Methods." FV x Rf is a parameter which is directly proportional to the amount of fused viral glycoproteins that become laterally mobile on the cell surface (16). All results are mean ± S.E.

| Viral protein | Treatment | D x 10^-10 | Rf | FV | FV x Rf | Cell fusion % |
|---------------|-----------|------------|----|-----|---------|---------------|
| F             | SConA     | 3.1 ± 0.2  | 0.62 ± 0.05 | 50 ± 4 | 31 | 68 ± 5 |
| F             | SConA + anti-ConA | 3.2 ± 0.3 | 0.54 ± 0.04 | 49 ± 4 | 26 | 56 ± 4 |
| HN            | SConA     | 3.3 ± 0.3  | 0.60 ± 0.04 | 50 ± 4 | 30 | 68 ± 5 |
| HN            | SConA + anti-ConA | 3.3 ± 0.3 | 0.55 ± 0.06 | 48 ± 4 | 26 | 56 ± 5 |

Fig. 4. Effects of anti-HN (A) and anti-F (B) IgG on virus-cell and cell-cell fusion. Erythrocytes were treated with virus and with the appropriate IgG prior to fusion (see "Materials and Methods"). Virus-cell fusion and cell-cell fusion were measured as described under "Materials and Methods" (see also Fig. 1). Results are mean ± S.E. of four separate experiments. Δ—Δ, virus-cell fusion (number of fused virions/cell). O—O, cell-cell fusion (% fused cells). ●—●, expected contribution of the reduction in virus-cell fusion to the inhibition of cell-cell fusion; values were calculated for each IgG concentration using Fig. 2 as a calibration curve, as in Fig. 1.

inhibition by SConA plus anti-ConA serum, and the calibration curve (Fig. 2) indicates that the reduction in the number of fused virions per cell could produce only a minor decrease (from 68% to about 60% fused cells) in cell-cell fusion. The level of cell-cell fusion appears to be in direct correlation with the concentration of laterally mobile viral glycoproteins in the target cell membrane, a parameter which is directly proportional to the product of the number of fused virions per cell

TABLE I

Effects of anti-F or anti-HN IgG on the lateral mobility of Sendai virus envelope proteins in the cell membrane following fusion

Erythrocytes agglutinated by Sendai virions were incubated with the indicated IgG (or with solution A where no antibody is indicated) at 19°C prior to fusion. After fusion at 37°C, they were labeled with either anti-F or anti-HN TMR-Fab' fragments (see "Materials and Methods"). FPR measurements were conducted on 50-60 cells in each case. Results are mean ± S.E.

| Viral protein | IgG | Rf | Cells with measurable Rf | D x 10^-10 |
|---------------|-----|----|--------------------------|-----------|
| F             | Anti-F (100 μg/ml) | 0.62 ± 0.05 | 98 ± 4 | 3.1 ± 0.2 |
| F             | Anti-F (50 μg/ml)  | 0.51 ± 0.06 | 85 ± 3 | 2.9 ± 0.3 |
| HN            | Anti-HN (100 μg/ml) | 0.60 ± 0.04 | 94 ± 4 | 3.3 ± 0.3 |
| HN            | Anti-HN (50 μg/ml) | 0.41 ± 0.06 | 79 ± 3 | 3.1 ± 0.3 |
| HN            | Anti-HN (100 μg/ml) | 0.00 ± 0.04 | 20 ± 3 | ND* |

* ND = not determined.

Effects of IgG-mediated Cross-linking of F or HN on Fusion and on Viral Glycoprotein Mobility—SConA cross-links and immobilizes both F and HN (Fig. 3, Table I). Thus, the experiments with SConA are not sufficient to discern whether the mobility of both viral glycoproteins or only one is required for the virally mediated fusion. To explore this question, we employed whole IgG directed against HN or against F to specifically cross-link each viral glycoprotein. After the agglutination step, erythrocytes were incubated (at 19°C, where fusion does not occur) with either anti-HN or anti-F IgG and warmed to 37°C to induce fusion (see "Materials and Methods"). Anti-HN IgG yielded a picture very similar to that observed with SConA (Fig. 4A). Increasing antibody concentrations inhibited both virus-cell and cell-cell fusion, the inhibition of the latter being significantly stronger. The actual inhibition of cell-cell fusion by anti-HN IgG is manyfold higher than that expected due to the mild reduction in the number of fused virions per cell (Fig. 4A); the expected inhibition was calculated using the calibration curve shown in Fig. 2, as was done (Fig. 1) in the case of SConA.

While the inhibition of virus-cell fusion by the anti-F IgG was rather similar to that induced by anti-HN IgG, the anti-F antibodies hardly inhibited cell-cell fusion, and the extremely weak inhibition of this parameter coincided closely with the level expected to be caused by the reduction in virus-cell fusion (Fig. 4B). This finding is in excellent correlation
Erythrocytes agglutinated with Sendai virions were incubated at 19 °C with anti-F or anti-HN IgG (20 μg/ml) followed by GAR IgG (50 μg/ml). In cases where GAR IgG and/or antiviral IgG were omitted, they were replaced by the incubation buffer. After fusion at 37 °C, they were labeled with anti-F or anti-HN TMR-Fab' fragments (designated under the column of Viral Protein) (detailed experimental protocol given under “Materials and Methods”). Rf and D were measured by FPR on 50–60 cells in each case. FV (fused virions/cell) and cell-cell fusion were determined in four separate experiments in each case, as described under “Materials and Methods.” FV × Rf is a parameter directly proportional to the concentration of laterally mobile viral glycoproteins on the cell surface (16). All results are mean ± S.E. Treatment with GAR IgG alone (without prior incubation with anti-F or anti-HN IgG) had no effect on any of the parameters measured.

| Viral protein | IgG          | D            | Rf          | FV | FV × Rf | Cell-cell fusion |
|---------------|--------------|--------------|-------------|----|---------|-----------------|
| HN            | Anti-HN      | 3.3 ± 0.3    | 0.60 ± 0.04 | 50 ± 4 | 30 | 68 ± 5         |
| HN            | Anti-HN + GAR IgG | 3.2 ± 0.4    | 0.48 ± 0.05 | 40 ± 4 | 19 | 52 ± 5         |
| F             | Anti-F       | 3.1 ± 0.2    | 0.62 ± 0.05 | 50 ± 4 | 31 | 68 ± 5         |
| F             | Anti-F + GAR IgG | 3.0 ± 0.4    | 0.59 ± 0.05 | 36 ± 4 | 21 | 67 ± 5         |
| HN            | Anti-F + GAR IgG | 3.4 ± 0.4    | 0.54 ± 0.04 | 34 ± 4 | 8  | 17 ± 4         |
| F             | Anti-HN + GAR IgG | 2.9 ± 0.4    | 0.55 ± 0.04 | 41 ± 3 |    |                 |

*Control experiments demonstrating that immobilization of F by anti-F IgG + GAR IgG does not affect significantly Rf of HN and vice versa. The slight reduction in Rf of the second viral glycoprotein (which was not cross-linked) is even less than the decrease in the number of fused virions/cell following the antibody treatment, most likely due to dissociation of some bound virions during the incubation with the antibodies (see text).

**Table III**

**Effects of anti-HN or anti-F IgG further cross-linked by GAR IgG on the lateral mobility of the viral glycoproteins and on Sendai virus-mediated fusion**

**Fig. 5.** Correlation between the level of laterally mobile viral glycoproteins in the cell membrane and cell-cell fusion. For example, the number of fused virions per cell times Rf of the F protein is directly proportional to the level of laterally mobile fused F proteins in the cell membrane (16). Data are compiled from Tables I and III. F and HN in the figure designate the viral glycoproteins which were labeled with TMR-Fab' fragments after fusion and whose Rf values were determined in each case. In the cases where antiviral IgG were employed to induce immobilization, the TMR-Fab' fragments used for the labeling and Rf measurements were derived from the same IgG used for cross-linking. In cases where SConA was employed, either F or HN were labeled, and their Rf values were measured separately. Δ, control (no treatment with either IgG or SConA). ○, incubation (prior to fusion) with 20 μg/ml antiviral glycoprotein IgG. □, incubation (prior to fusion) with 1.5 μg/ml SConA. ●, incubation with 20 μg/ml antiviral glycoprotein IgG followed by incubation with 50 μg/ml GAR IgG. ■, incubation with 1.5 μg/ml SConA followed by incubation with a 1:20 dilution of anti-ConA antiserum.

with the effects of the two antibodies on the lateral mobilities of the viral glycoproteins against which they are directed (Table II): while the anti-HN IgG reduced the Rf levels of the HN proteins, the anti-F IgG hardly affected Rf of the F proteins even at a high dose. The partial inhibition of virus-cell fusion by the anti-F IgG, which is very similar to that induced by the anti-HN IgG, indicates that it does bind to the cell-adherent virions; therefore, the difference between the two IgG preparations in the effects on viral glycoprotein mobility and cell-cell fusion most likely reflects differences in the accessibility of the antigenic sites on F and HN proteins embedded in the viral envelope (prior to fusion) to the respective antibodies, resulting in a lower cross-linking capability of the anti-F IgG. It should be noted that the Rf values for the viral glycoproteins following cross-linking with IgG may be even lower than those depicted in Table II, since antigenic sites already occupied by the IgG are not available for labeling with TMR-Fab' fragments derived from the same IgG; a possible result is preferential labeling of those viral glycoproteins that bound fewer IgG molecules. However, this effect appears to be negligible in the case of anti-F IgG (100 μg/ml) and up to 30 μg/ml anti-HN IgG, since only 10% of the sites available for labeling with TMR-Fab' on erythrocytes fused with the virions were blocked by incubation with the respective IgG molecules after the agglutination step (labeling was measured by the FPR instrumentation under nonbleaching conditions). Even in the case of incubation with 100 μg/ml anti-HN IgG, only 30% of the sites were blocked.

The failure of the anti-F IgG to immobilize the F proteins (and to inhibit cell-cell fusion) necessitated the application of a second antibody layer (GAR IgG) to enhance the cross-linking of the F proteins. In the case of the anti-HN IgG, treatment with a low IgG concentration followed by GAR IgG can also be employed to minimize the possible contribution of direct binding of the first antibody to the inhibition of cell-cell fusion and measure effects which are exclusively due to cross-linking. In these experiments, erythrocytes agglutinated by Sendai virions were incubated with 20 μg/ml anti-F or anti-HN IgG—a concentration which has a weak effect on cell-cell fusion in the case of anti-HN IgG, and no effect at all in the case of anti-F IgG (Fig. 4, Table III). This was followed by further cross-linking by GAR IgG, fusion at 37 °C, and labeling with anti-F or anti-HN TMR-Fab' fragments (see “Materials and Methods”). The results (Table III) demonstrate that further cross-linking of the viral glycoproteins by GAR IgG immobilizes the viral glycoproteins and leads to a marked reduction in cell-cell fusion. This effect is seen with
both F and HN, further reinforcing the notion that the failure of the anti-F IgG by itself to immobilize the F proteins and inhibit cell-cell fusion is due to a low level of cross-linking rather than due to lack of binding. As in the case of fusion inhibition by SConA, the level of cell-cell fusion is clearly correlated with the number of fused virions per cell multiplied by $R_f$, a product which is directly proportional to the amount of fused viral glycoproteins that become laterally mobile on the cell surface (Table III, Fig. 5).

Table III also depicts experiments which demonstrate that there is no significant cross-reactivity between the anti-HN and anti-F IgG preparations under the experimental conditions employed in the FPR and in the fusion-inhibition experiments. Thus, incubation with anti-HN IgG followed by GAR IgG resulted in only a very slight decrease in $R_f$ of the F proteins and vice versa. It should be noted that one does not expect a total lack of effect of cross-linking, e.g., HN on the lateral mobility of F, since the IgGs reduce virus-cell fusion, and envelope proteins of unfused virions bound to the cells appear as immobile in the FPR experiments (12, 34). The slight reduction in $R_f$ of one viral glycoprotein upon cross-linking of the other is even below the decrease in the number of fused virions per cell, most likely because part of the adsorbed unfused virions dissociate from the cells during the incubation at 19°C as a result of the viral neuraminidase activity (35); the dissociated virions do not contribute to the FPR measurements, which focus on the cell membrane.

**DISCUSSION**

The current work was aimed to investigate the effects of immobilization of Sendai virus envelope proteins on the virally meditated fusion. The results demonstrate that cross-linking of the viral glycoproteins (after adsorption but prior to fusion) by SConA (Figs. 1 and 3; Table I) or by IgG directed against F or HN (Fig. 4; Tables II and III) inhibits cell-cell fusion whenever a significant fraction of the viral glycoproteins is immobilized. On the other hand, the binding of an antibody which does not induce enough cross-linking to immobilize its antigen (as in the case of treatment with anti-F IgG) also does not lead to a significant inhibition of cell-cell fusion (Fig. 4, Table II). Since both SConA and the antiviral IgG act through binding to the viral glycoproteins, part of their inhibitory effects on cell-cell fusion could be contributed by the binding itself (either due to binding to a region essential for the fusogenic activity, or by a steric effect interfering with the ability of the fusogenic protein to come into close contact with the target membrane). In order to minimize the contribution of direct binding, the viral glycoproteins were cross-linked with low concentrations of SConA or antiviral IgG, chosen to induce minimal effects on their lateral mobility or on fusion. A high degree of cross-linking leading to viral glycoprotein immobilization was achieved by the use of a second antibody (anti-ConA or GAR IgG). Since the secondary antibodies by themselves do not inhibit fusion (either cell-cell or virus-cell), their effects are exclusively due to cross-linking the prebound SConA or antiviral IgG and not through direct binding to the viral glycoproteins. These experiments (Tables I and III) strongly suggest that immobilization of the viral glycoproteins eliminates their ability to induce cell-cell fusion, and that the contribution of direct binding to the inhibition of cell-cell fusion under these conditions is minor. This notion is further supported by the demonstration that the virus-cell fusion activity is still high (60–80% of control) even under the double cross-linking conditions that strongly inhibit cell-cell fusion (Tables I and III); since virus-cell and cell-cell fusion are induced by the same viral glycoproteins, direct binding of the antibodies or SConA is expected to inhibit both processes.

If the inhibition of cell-cell fusion is due to immobilization of the viral glycoproteins, a strict correlation should be found between the level of cell-cell fusion and the concentration of laterally mobile viral glycoproteins in the target cell membrane. Fig. 5 demonstrates such a correlation. Interestingly, a given reduction in the number of fused virions times $R_f$ (the parameter proportional to the level of laterally mobile molecular complexes of the protein whose $R_f$ was measured) results in a stronger inhibition of cell-cell fusion in the case of SConA as compared with anti-F or anti-HN IgG (Fig. 5). This most likely reflects the fact that each antiviral IgG inhibits the motion of only one viral glycoprotein type, while SConA immobilizes both F and HN. This notion is in accord with the effects of suboptimal fusion temperatures on $R_f$ of the viral glycoproteins and on cell-cell fusion (16): fusion temperatures below 31°C lead to a concomitant reduction in $R_f$ of both F and HN, and the dependence of cell-cell fusion on the number of fused virions times $R_f$ was essentially identical with that observed using SConA to inhibit cell-cell fusion.

The inhibition of cell-cell fusion following specific immobilization of either F or HN by the appropriate IgG suggests that lateral mobility of both F and HN is required for cell-cell fusion. An immediate conclusion from the requirement for HN mobility is that aside of its role in virus binding and cell agglutination, this protein also has a role in the induction of cell-cell fusion. This conclusion is in line with several earlier studies, which suggested the possibility that HN is needed for the fusogenic activities (7–11).

The exact role of the lateral mobility of the viral glycoproteins in the fusion mechanism and the reasons for the much higher sensitivity of cell-cell fusion as compared with virus-cell fusion to the inhibition of viral glycoprotein mobility are not yet clear. It is possible that lateral diffusion of the viral envelope proteins in the cell membrane is required to enable several such proteins to associate into an active fusogenic entity, while on the intact virion their dense packing may result in a spatial arrangement which enables the formation of an active fusogenic complex without the need for lateral motion within the viral envelope. An analogous interpretation was suggested by Morris et al. (36) to explain the much longer lag time observed for cell-cell fusion mediated by the influenza hemagglutinin protein expressed in transfected cells as compared with the very short lag time in virus-cell fusion. An alternative explanation based on differences in viral glycoprotein density (but which does not assume a specific fusion complex) is that the density of the fusogenic proteins has to exceed a certain minimal level in membrane contact regions in order to induce fusion; in that case, lateral motion of the viral glycoproteins in the target cell membrane could be required to enable them to achieve high concentrations in cell-cell contacts. On the other hand, the high density of the viral envelope proteins on the virion itself may reduce the need for such motion in the case of virus-cell fusion, resulting in a significantly lower sensitivity of this parameter to lateral immobilization of the viral glycoproteins.

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