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Use of Virus-Attached Antibodies or Insulin Molecules To Mediate Fusion between Sendai Virus Envelopes and Neuraminidase-Treated Cells†

A. G. Gitman,‡ I. Kahane,§ and A. Loyter*,‡

Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel, and Department of Membrane and Ultrastructure Research, Hadassah Hospital-Hebrew University, Ein Karem, Jerusalem, Israel

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ABSTRACT: Anti-human erythrocyte antibodies or insulin molecules were covalently coupled to the glycoproteins (the hemagglutinin/neuraminidase and the fusion polypeptides) of Sendai virus envelopes with *N*-succinimidyl 3-(2-pyridyldithio)propionate and succinimidyl 4-(*p*-maleimidophenyl)butyrate as cross-linking reagents. Reconstituted Sendai virus envelopes, bearing covalently attached anti-human erythrocyte antibodies or insulin molecules, were able to bind to but not fuse with virus receptor depleted human erythrocytes (neuraminidase-treated human erythrocytes). Only coreconstitution of Sendai virus glycoproteins, bearing attached anti-human erythrocyte antibodies or insulin molecules with intact, untreated viral glycoproteins, led to the formation of fusogenic, targeted reconstituted Sendai virus envelopes. Binding and fusion of reconstituted Sendai virus envelopes, bearing anti-human erythrocyte antibodies or insulin molecules, with neuraminidase-treated human erythrocytes were blocked by the monovalent fraction, obtained after papain digestion of immunoglobulins, made of anti-human erythrocyte antibodies or free insulin molecules, respectively. The results of this work demonstrate an active role of the viral binding protein (hemagglutinin/neuraminidase polypeptide) in the virus membrane fusion process and show a novel and efficient method for the construction of targeted, fusogenic Sendai virus envelopes.

Resealed vesicles made of pure phospholipid molecules (liposomes) (Papahadjopoulos et al., 1982) or fusogenic reconstituted Sendai virus envelopes (RSVE)[1] (Loyter & Volsky, 1982) have been used during the past few years as an efficient biological carrier for the introduction of otherwise impermeable molecules into living cells. RSVE have been also shown to be powerful vehicles for the implantation of various membrane components such as virus, lectin, or hormone receptors into

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‡ Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem.

§ Department of Membrane and Ultrastructure Research, Hadassah Hospital-Hebrew University.

[1] Abbreviations: IgG, immunoglobulin G; F(ab), monovalent fraction obtained after papain digestion of IgG; RSVE, reconstituted Sendai virus envelopes; HE, human erythrocytes; PMSF, phenylmethanesulfonyl fluoride; anti-HE, anti-human erythrocyte antibodies; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; SMPB, succinimidyl 4-(*p*-maleimidophenyl)butyrate; DTT, dithiothreitol; HN, hemagglutinin/neuraminidase glycoprotein of Sendai virus; F, fusion factor; polypeptide–MPB, polypeptide–(maleimidophenyl)butyrate; polypeptide–PDP, polypeptide–(pyridyldithio)propionate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; anti-HE-F(ab), F(ab) fraction of anti-human erythrocyte antibodies; Tris, tris(hydroxymethyl)aminomethane.

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plasma membranes of living cells (Loyter & Volsky, 1982; Prujansky-Jacobovitz et al., 1981). In the light of these observations, an extensive effort is being made to construct stable vehicles that potentially will be able to carry drugs, enzymes, or functional genes into specific cells in whole animals. Indeed, several drugs, such as the antileishmania drug (meglumine antimoniate) (Chapman et al., 1984), have been shown to be less toxic and more efficient, if they were administered into animals via loaded liposomes. Recently, liposomes loaded with DNA molecules have been able to carry genes coding for insulin into laboratory mice (Nicolau et al., 1983).

An ideal vehicle for the introduction of macromolecules and drugs into both cultured cells and whole animals would be a fusogenic vesicle that will introduce its content directly into the cytoplasm of specific cells. Envelopes of Sendai virions (Loyter & Volsky, 1982) or fusogenic liposomes (Garcia et al., 1984) may, after some modifications, offer to be such a vehicle. However, both phospholipid liposomes or RSVE interact with a wide spectrum of cells. Liposomes attach mainly unspecifically to cell plasma membranes (Poste, 1980), while Sendai virus envelopes interact with membrane sialic acid residues abundant in membranes of most eukaryotic cells (Poste & Pasternak, 1978).

During recent years, efforts have been made to couple specific antibodies or other ligands to phospholipid liposomes in order to construct a specific "targeted" liposome. It seems that the most efficient way is to chemically couple IgG or F(ab)′ molecules to the amino groups of phospholipids via cross-linking reagents such as SPDP or SMPB (Martin et al., 1982). Indeed, liposomes bearing specific antibodies interacted more effectively with cells carrying the appropriate antigens (Martin et al., 1982).

Constructing a targeted RSVE is a more complicated process. Such a method should result in neutralization of the Sendai virus binding protein, namely, the HN glycoprotein, while preserving the virus fusogenic activity. Guyden et al. (1983) constructed fusogenic, targeted Sendai virions by chemically coupling biotin molecules to the virus particles and avidin molecules to membranes of recipient cells. However, this method is impractical for in vivo use because for attachment of the avidin molecules the recipient cells must be isolated. Heath et al. (1983) have constructed antibody-bearing Sendai virions by incorporating a conjugate of ganglioside–IgG into envelopes of intact Sendai virus particles. The gangliosides that are incorporated into the virus envelopes during incubation at 37 °C leave the IgG molecules exposed on the virus surface. Such targeted Sendai virions, bearing anti-human erythrocyte antibodies (anti-HE-Ab), were able to interact and to fuse with neuraminidase-treated human erythrocytes (HE) (Heath et al., 1983).

In our laboratory we have used a similar approach to insert specific antibodies into Sendai virus envelopes (Gitman & Loyter, 1984). IgG molecules were chemically coupled to the thio-containing parafin molecule dodecanethiol. The conjugate dodecanethiol–IgG was then added to a detergent solution of the Sendai virus envelope phospholipids and glycoproteins. Removal of the detergent led to the formation of reconstituted envelopes containing the dodecanethiol–antibody complex (Gitman & Loyter, 1984).

It is clear that, in contrast to the use of the couple avidin–biotin (Guyden et al., 1983), the above two methods, namely, the use of ganglioside–IgG (Heath et al., 1983) or dodecanethiol–IgG (Gitman & Loyter, 1984) conjugates, required modification of the viral envelopes only, without treatment of cell plasma membranes. However, these methods need the addition of new hydrophobic molecules such as the ganglioside or the dodecanethiol to the virus envelopes. These molecules, at high concentrations, may affect the viral fusogenic activity and, therefore, limit the number of antibody molecules that can be inserted into the viral envelopes. Furthermore, it is not unreasonable to assume that, when such targeted vesicles will be used in vivo after intravenous injection, both the ganglioside and the dodecanethiol conjugates may be removed from the viral envelopes by serum–lipoprotein complexes.

An alternative way to construct targeted fusogenic Sendai virus envelopes would be to couple specific ligands to the viral envelope glycoproteins (spikes). In contrast to membrane gangliosides or phospholipids, which are buried within the viral membrane, the viral glycoproteins are exposed on the viral surface, having a length of 70 Å (Loyter & Volsky, 1982). Therefore, it is conceivable that, in addition to large molecules, also ligands of small molecular weight will be useful as recognition proteins, if they will be coupled to the viral spikes.

We have previously shown that IgG molecules can be covalently coupled to the glycoproteins of intact Sendai virions (Tomasi et al., 1982). Although such antibody-bearing virus particles were able to attach to virus receptor depleted human erythrocytes, they showed very low fusogenic activity (Tomasi et al., 1982). This may be due to the fact that the two viral envelope polypeptides, e.g., the HN and the F glycoproteins, were inactivated by the chemical modification (reduction with DTT) required for the coupling reaction.

In the present work, specific anti-HE-Ab and insulin molecules were coupled to the viral envelope glycoproteins after they had been solubilized with Triton X-100. Coreconstitution of the IgG or the insulin-bearing glycoproteins with untreated, active Sendai virus glycoproteins resulted in the formation of highly fusogenic, targeted RSVE.

Experimental Procedures

Virus. Sendai virus was propagated, harvested, and suspended in solution A (150 mM NaCl, 20 mM Tris, pH 7.4) to give 10% (v/v), and its hemagglutinating units were determined as before (Toister & Loyter, 1973). Virus-induced agglutination and hemolysis of human red blood cells were determined as previously described (Toister & Loyter, 1973).

Cells. Human blood, type O, R H^+, recently outdated, was washed 3 times in solution A, and the final pellet was resuspended in the same buffer to give 2.5% (v/v). The HE were desialyzed by neuraminidase, as described earlier (Lalazar et al., 1977).

Preparation of Polypeptide–MPB (or –PDP) Complexes. To a solution containing either anti-HE-Ab, insulin, human IgG, or BSA (2–3 mg/500 μL of PBS), SMPB or SPDP (1–2 mg/100 μL of dimethyl sulfoxide) was added, to give a molar ratio of protein:SMPB (or SPDP) of 1:5. After 1 h of incubation at 20 °C, the solution obtained (about 500 μL) was loaded on a 5-mL Sephadex G-25 column packed in a 5-mL syringe. Polypeptide–MBP (or –PDP) was eluted by centrifugation at 1200*g* for 3 min. Recovery of the polypeptides was determined either by estimating protein concentration, with the method of Lowry et al. (1951), or by the use of radioiodinated polypeptides.

Binding of Polypeptide–MPB (or –PDP) to DTT-Reduced, Detergent-Solubilized Sendai Virus Glycoproteins. Sendai virus particles were dissolved in Triton X-100, essentially as described before (Vainstein et al., 1984). Breifly, a pellet containing 10 mg of Sendai virus particles was dissolved in 1 mL of 2% (v/v) Triton X-100, and after removal of detergent-insoluble material, the pH of the clear supernatant, which contained 2–2.5 mg of the viral envelope glycoproteins, was adjusted to 8.0 by the addition of 1 M of NaOH. DTT (100 mM in solution A, pH 8.2) was added to give a final con-

centration of 3 mM. After 60 min at 37 °C, unreacted DTT was removed by dialyzing the detergent solution against solution A for 4–5 h at 4 °C with three changes of the external buffer.

Covalent binding of polypeptide–MPB (or –PDP) to the DTT-reduced viral glycoproteins was performed as follows: To each 500 μL of a detergent solution containing DTT-reduced viral glycoproteins (2–2.5 mg/mL), 1 mg of polypeptide–MPB (or –PDP) was added. After 60 min at 37 °C, the detergent (Triton X-100) was removed by the direct addition of SM-2 Bio-Beads, as described before (Vainstein et al., 1984). The turbid suspension obtained contained RSVE bearing covalently bound polypeptides (modified viral glycoproteins). After centrifugation at 100000*g* for 1 h at 4 °C, the pellet obtained was suspended in 300 μL of solution A (to give about 3–3.5 mg/mL). The concentration of the viral glycoproteins was calculated by subtracting the amount of the attached polypeptides (which was determined by using radioiodinated polypeptides) from the toal amount of protein, as estimated by the method of Lowry et al. (1951).

Construction of Active, Fusogenic RSVE Bearing Covalently Bound Polypeptides. Detergent solutions of modified and intact, unmodified viral glycoproteins (each containing 2–2.5 mg of viral protein/mL) were mixed, if not otherwise stated, in a volume ratio of 1:6, respectively. Triton X-100 was removed from the mixture by direct addition of SM-2 Bio-Beads, exactly as described before (Vainstein et al., 1984). After centrifugation (100000*g*, 1 h at 4 °C), the pellet obtained was dissolved in 300 μL of solution A, to give about 3–3.5 mg of viral protein/mL. In cases where high amounts of detergent-solubilized viral glycoproteins were needed, numerous pellets of 10 mg of Sendai virus particles were dissolved simultaneously. Attempts to dissolve high amounts of Sendai virus particles met with difficulties and resulted in inefficient solubilization.

Rabbit anti-HE-Ab were obtained by subcutaneous injection of erythrocyte ghosts, as described before (Gitman & Loyter, 1984). The IgG fraction was obtained by ammonium sulfate precipitation (33% v/v) (Heide & Schwick, 1978). F(ab) fragments of anti-HE-Ab were obtained following papain hydrolysis of the IgG fraction, as previously described (Garvey et al., 1977). Sendai virus, human IgG, anti-HE-Ab, and insulin were labeled with $Na^{125}I$ and iodogen, as described earlier (Markwell & Fox, 1978). ^{125}I-RSVE was obtained by solubilization of ^{125}I-labeled Sendai virions. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Materials. SPDP and SMPB were purchased from Pierce; DTT, human IgG (fraction II), and bovine insulin were from Sigma; neuraminidase from *Vibrio cholera* (500 units/mL) was obtained from BDH Biochemicals.

Results

Construction of Hemolytic Targeted RSVE: Requirement for Intact Viral Glycoproteins. The results in Table I show that anti-HE-Ab can be coupled to RSVE. This can be achieved by the addition of either MPB-anti-HE-Ab or PDP-anti-HE-Ab to a detergent solution of DTT-reduced viral glycoproteins. As can be seen, the cross-linking reagents SPDP and SMPB mediated covalent binding of the IgG molecules to the viral glycoproteins. The view that disulfide bonds mediate binding between the IgG molecules and the viral glycoproteins is evident from the results showing that DTT removed IgG molecules attached via PDP but not via MPB (Table I). The dithio bonds established by SPDP are reversible, while the thioether bonds formed by SMPB are not.

Table I: Covalent Coupling of ^{125}I-Anti-HE-Ab to DTT-Reduced Detergent-Solubilized Sendai Virus Envelope Glycoproteins[a]

system	anti-HE-Ab bound/ viral glycoprotein (μg/μg)
(A) added to a detergent solution of DTT-reduced viral glycoproteins	
MPB–anti-HE-Ab	0.21
PDP–anti-HE-Ab	0.19
anti-HE-Ab	0.05
(B) treatment with DTT of	
RSVE–MPB–anti-HE-Ab	0.18
RSVE–PDP–anti-HE-Ab	0.05

[a] Anti-HE-Ab were prepared and radioiodinated as described under Experimental Procedures, to give a specific radioactivity of 3×10^4 cpm/μg of protein. The ^{125}I-IgG molecules were then reacted with SMPB or SPDP, and the resulting MPB–IgG and PDP–IgG were mixed with detergent-solubilized DTT-reduced viral glycoproteins, as described under Experimental Procedures. After removal of the detergent (Triton X-100), the vesicles obtained (300 μL) were washed once with solution A (7 mL), and samples (100 μL) were withdrawn for estimation of radioactivity (A). The remainder (200 μL of a suspension containing either RSVE–MPB–anti-HE-Ab or RSVE–PDP–anti-HE-Ab) was reduced with DTT (10 mM final concentration) for 45 min at 37 °C. At the end of the incubation period, the reduced RSVE were collected and centrifuged (100000*g*, 40 min), and after being washed with solution A (7 mL), their radioactivity was determined by gamma counter.

Membrane sialic acid residues are known to serve as specific receptors for Sendai virus particles (Poste & Pasternak, 1978). Table II shows that RSVE, bearing anti-HE-Ab, are able to attach to desialized HE. This is evident from the ability of antibody-bearing virus particles to cause agglutination of the desialized HE (Table II, experiment 1). However, RSVE bearing the anti-HE-Ab failed to hemolyze desialized HE. It is noteworthy that virus-induced hemolysis reflects a process of virus cell fusion (Maeda et al., 1977). Hemolytic targeted RSVE were obtained only when a detergent solution of viral glycoproteins, bearing antibodies (modified viral glycoproteins) were mixed with unmodified viral glycoproteins. Removal of the detergent led to the formation of reconstituted vesicles containing unmodified and modified viral glycoproteins within the same membrane. As can be seen (Table II, experimental 1), such "hybrid" vesicles were able to induce hemolysis in desialized HE, the degree of which was dependent upon the ratio between modified to modified glycoproteins.

Similar conclusions can be also inferred from the results in Table II, experiment 2, in which increasing amounts of modified viral glycoproteins were coreconstituted with a constant amount of unmodified viral glycoproteins. The most active vesicles (high degree of hemolysis per microgram vesicle) were obtained with RSVE containing low amounts of modified and high amounts of unmodified viral glycoproteins.

The requirement for unmodified viral glycoproteins (the viral HN and F polypeptide) to obtain functional (hemolytic and fusogenic) targeted RSVE, is also evident from the results in Table III. These results show that RSVE-bearing antibodies were able to agglutinate but not to hemolyze desialized HE. On the other hand, vesicles containing either intact (unmodified) HN or F glycoproteins neither agglutinated (bound to) nor hemolyzed desialized HE. As can be seen in this table, coreconstitution of the viral-modified glycoproteins, together with either the HN or the F polypeptide, did not alter their properties, and such vesivles could only agglutinate but not hemolyze desialized HE. Only coreconstitution of the modified viral glycoproteins, together with both intact HN and F polypeptides led to the formation of hemolytic (fusogenic) targeted viral envelopes.

Interaction between Targeted RSVE and Desialized HE:

Table II: Induction of Hemolysis in Desialized HE by RSVE-Bearing Anti-HE-Ab: Requirement of Intact, Unmodified Viral Glycoproteins[a]

	detergent solution of		anti-HE-Ab/viral glycoprotein (μg/μg)	agglutination	hemolysis (% of total)
	viral glycoproteins–MPB–anti-HE-Ab (μL)	unmodified viral glycoproteins (μL)			
expt 1	500	0	0.40	+	6
	500	50	0.36	+	29
	500	100	0.26	+	40
	500	500	0.18	+	49
expt 2	0	300[b]	0.05[b]	–	8
	50	300	0.20	+	63
	200	300	0.50	+	36
	600	300	0.40	+	35
	200	0	0.88	+	4

[a] Sendai virus particles were dissolved in Triton X-100, and their detergent-solubilized glycoproteins were reduced by DTT and coupled to radioiodinated MPB–anti-HE-Ab (6×10^5 cpm/μg of protein), as described under Experimental Procedures. Increasing volumes of unmodified, detergent-solubilized viral glycoproteins (3.1 mg/mL viral glycoproteins) were then added to 500 μL of the modified, detergent-solubilized viral glycoproteins (glycoprotein–MPB–Ab, 3.1 mg of viral glycoprotein/mL) (experiment 1). In experiment 2, increasing volumes of the modified viral glycoproteins were added to 300 μL of a detergent solution of the unmodified viral envelope glycoproteins. After removal of the detergent, the radioactivity associated with the RSVE formed was estimated. Samples of 10 μg of RSVE were then incubated with 200 μL of 2.5% (v/v) desialized human red blood cells. Agglutination was observed and estimated by phase microscopy, and hemolysis was determined after 30 min of incubation at 37 °C (Toister & Loyter, 1973). [b] In this experiment only radioiodinated MPB–anti-HE-Ab were added to unmodified detergent-solubilized viral glycoproteins.

Table III: Requirement for Active HN and F Glycoproteins for Construction of Targeted Hemolytic (Fusogenic) RSVE[a]

detergent solution of				
modified viral glycoproteins (μL)	unmodified viral glycoproteins (μL)		agglutination	hemolysis (% of total)
	HN	F		
300	0	0	+	7
0	500	0	–	8
0	0	500	–	7
300	500	0	+	10
300	0	500	+	4
300	500	500	+	45
0	500	500	–	6

[a] The viral HN and F glycoproteins were purified by using DEAE-cellulose and CMC-Sepharose, respectively, as described elsewhere (Nussbaum et al., 1984). Detergent-solubilized HN and F glycoproteins (800 μg of protein of each per milliliter) were mixed with modified glycoproteins (glycoproteins–MPB–anti-HE-Ab, 3 mg/mL) at the volume ratios indicated in the table. After removal of the detergent, the reconstituted envelopes formed (5 μg) were incubated with 200 μL of 2.5% (v/v) desialized HE.

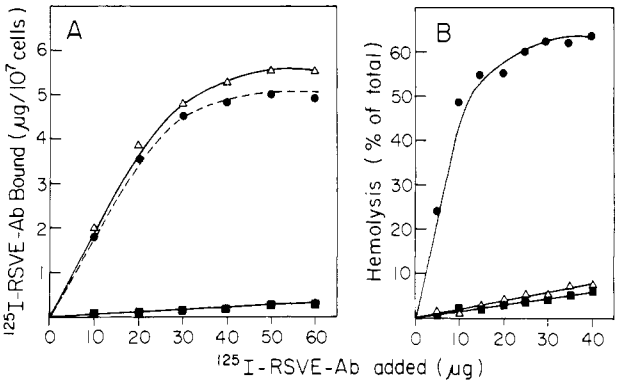

FIGURE 1: Interaction of antibody-bearing RSVE with desialized HE. Binding of anti-HE-Ab and human IgG molecules (using SMPB as a cross-linking reagent) to detergent-solubilized ^{125}I-labeled viral envelope glycoproteins and reconstitution of hemolytic Sendai virus envelopes (ratio of unmodified to modified viral glycoprotein in the reconstitution system, 6:1) were as described under Experimental Procedures. Increasing concentrations of either RSVE-bearing anti-HE-Ab, reconstituted in the presence (●) or absence (△) of unmodified, intact viral glycoproteins of RSVE (containng unmodified viral glycoproteins) bearing human IgG molecules (■) were incubated with 10^7 desialized HE, in a final volume of 200 μL of solution A. Binding was determined after 40 min of incubation at 20 °C, a temperature at which very little, if any, hemolysis was induced. At the end of the incubation period, bound virus particles were separated from the erythrocyte-associated virus particles by centrifugation through a cushion of 0.3 M sucrose in Eppendorf tubes (Gitman & Loyter, 1984). The pellet obtained was washed twice with 1 mL of solution A, and after the bottom of the tubes was cut, the radioactivity associated with the pellet was estimated (A). Degree of hemolysis (B) was estimated at 540 nm, after 40 min of incubation at 37 °C, as before (Toister & Loyter, 1973).

Dependence on the Presence of Specific Antibody. The results in Figure 1A show that indeed reconstituted envelopes, bearing anti-HE-Ab, bind to desialized HE, reaching saturation at 30–40 μg of vesicles/10^7 cells. As can be seen in the figure, the binding characteristics of vesicles, containing only modified glycoproteins or a mixture of modified and unmodified viral glycoproteins, were the same (Figure 1A). However, only hybrid vesicles, containing both modified and unmodified viral glycoproteins, were able to induce hemolysis in desialized HE (Figure 1B). Vesicles bearing human IgG (instead of anti-HE-Ab) neither bound to nor caused hemolysis of desialized HE (Figure 1).

The requirement for specific anti-HE-Ab for obtaining functional targeted RSVE is also evident from the results in Figure 2. F(ab) fragments obtained from anti-HE-Ab were able to compete with the binding of RSVE-bearing antibodies to desialized HE (Figure 2B). Evidently, reduction in binding resulted in inhibition of the hemolytic activity of the targeted RSVE (Figure 2B). F(ab) fragments were also able to displace already bound, targeted RSVE (Figure 2D). Competition and displacement were specifically caused by F(ab) fragments obtained from anti-HE-Ab, since F(ab) fragments obtained from human IgG neither reduced the binding of nor caused hemolysis induced by RSVE-bearing anti-HE-Ab (Figure 2C). Specificity is also evident from the results showing that covalently attached human IgG (or BSA, not shown) did not substitute covalently attached anti-HE-Ab. RSVE-bearing human IgG neither bound to nor induced hemolysis of desialized HE Figure 2E).

The results in Table IV clearly show that the hemolytic activity of the targeted RSVE is due to the presence of the viral fusion (F) glycoprotein. Reconstituted envelopes that were treated with PMSF did not cause hemolysis of either control or neuraminidase-treated HE. Treatment with PMSF has previously been shown to affect only the viral hemolytic and fusogenic activity, without interfering with its binding

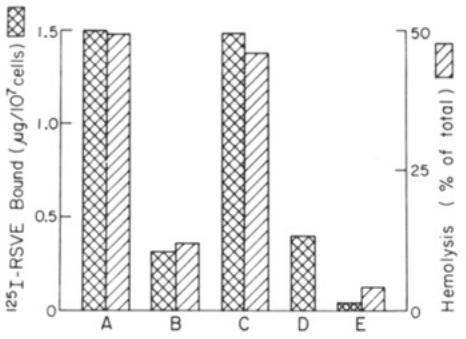

FIGURE 2: Effect of anti-human erythrocyte F(ab) molecules on the binding and hemolytic activities of RSVE-bearing antibodies. RSVE-bearing anti-HE-Ab or human IgG were obtained by mixing detergent solutions of unmodified and modified viral glycoproteins in a 3:1 weight ratio, respectively. RSVE-Ab (A) or RSVE–human IgG (E) (10 μg) were incubated with 10^7 desialized HE. In systems B and C, 200 μg of anti-HE-F(ab) and F(ab) obtained from human IgG, respectively, were incubated with the desialized HE (30 min at 20 °C) prior to the addition of the RSVE-Ab. In system D, 200 μg of anti-HE-F(ab) was added at the end of the incubation period (40 min at 20 °C) to a suspension containing RSVE-Ab and desialized HE. The suspension was allowed to incubate with the anti-HE-F(ab) molecules for another 30 min at 20 °C. Binding of RSVE-Ab (cross-hatched) and RSVE-Ab-induced hemolysis (hatched) of desialized HE were performed and determined as described in Figure 1.

Table IV: Virus-Induced Hemolysis of Desialized Human Erythrocytes: Requirement for Specific Anti-HE-Ab and Viral Fusogenic Activity[a]

	human erythrocytes		desialized human erythrocytes	
system	agglutination	hemolysis (% of total)	agglutination	hemolysis (% of total)
RSVE	+	81	–	6
RSVE–anti-HE-Ab	+	89	+	49
RSVE–anti-HE-Ab + PMSF	+	9	+	10
RSVE–human IgG	+	89	–	8

[a] Hemolytic RSVE–anti-HE-Ab were obtained by mixing detergent-solubilized, modified viral glycoproteins with unmodified detergent-solubilized viral glycoproteins. RSVE–human IgG were obtained in the same way, except that MPB–human IgG were attached to the viral glycoproteins instead of anti-HE-Ab. RSVE–anti-HE-Ab were incubated with PMSF (7 mM, final concentration), as described before (Israel et al., 1983). Agglutination and hemolysis were promoted by incubating 10 μg of RSVE with 200 μL of 2.5% (v/v) untreated and desialized HE.

ability (Israel et al., 1983). Furthermore, the results in Table IV show that covalent attachment of IgG molecules to the viral envelope did not, by itself, affect the viral biological activities. RSVE-bearing human IgG were able to bind to (agglutinate) and hemolyze control (but not desialized) HE to the same extent as untreated RSVE.

Covalent Attachment of Insulin Molecules to Sendai Virus Envelopes: Use of Insulin Molecules To Mediate Functional Binding between Virus Envelopes and Desialized HE. The results in Table V show that, similar to IgG molecules, insulin molecules can covalently be attached to RSVE. As can be seen, the concentration of the virus-associated insulin molecules (micrograms of insulin bound per microgram of viral glycoproteins) was dependent upon the amount of the complex MPB–insulin added to the detergent solution of the DTT-reduced viral glycoprotein (Table V). Very little, if any, insulin molecules were found associated with the RSVE either when free insulin molecules were added to the DTT-reduced viral glycoproteins when MPB–insulin complex was added to

Table V: Covalent Coupling of ^{125}I-Insulin to DTT-Reduced Sendai Virus Envelope Glycoproteins[a]

system	insulin-bound/ viral glycoprotein (μg/μg)
(A) added to a detergent solution of DTT-reduced viral glycoproteins	
MPB–insulin (0.5 mg)	0.30
MPB–insulin (1 mg)	0.67
insulin	0.06
(B) added to a detergent solution of unreduced viral glycoproteins	
MPB–insulin (0.5–1 mg)	0.05
insulin	0.05

[a] Insulin was radiolabeled with iodogen, to give a specific activity of (3–5) × 10^4 cpm/μg of protein. The ^{125}I-insulin was reacted with SMPB, and the resulting MPB–insulin was then mixed with detergent-solubilized, DTT-reduced viral envelope glycoproteins, as described under Experimental Procedures.

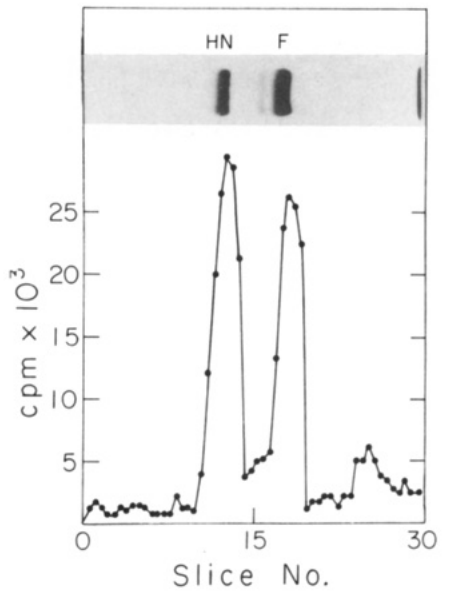

FIGURE 3: Covalent attachment of insulin molecules to Sendai virus HN and F glycoproteins: gel electrophoresis analysis. ^{125}I-Insulin was covalently attached to the Sendai virus envelope glycoproteins as described under Experimental Procedures and in Table V. The RSVE-bearing ^{125}I-insulin (100 μg of viral glycoproteins) was electrophoresed on SDS–PAGE (7.5% acrylamide) (Laemmli, 1970). For estimation of radioactivity associated with the viral glycoproteins, the gels were sliced (2-mm slices), and the radioactivity associated with the various slices was estimated by a gamma counter.

a detergent solution of unreduced Sendai virus glycoproteins (Table V). A gel electrophoresis analysis (Figure 3) of RSVE bearing ^{125}I-insulin revealed that, under the conditions used, the insulin molecules were attached almost to the same extent to the two viral envelope polypeptides, namely, the HN and the F glycoproteins.

It has been well documented that HE membranes possess exposed functional insulin receptors (Gambhir et al., 1978). Treatment of HE with neuraminidase does not significantly affect these receptors. The results in Figure 4A show that insulin molecules covalently coupled to RSVE can mediate attachment of ^{125}I-RSVE to desialized HE. However, RSVE that did not contain unmodified glycoproteins, despite their ability to attach to desialized HE, failed to cause hemolysis (Figure 4B). Only hybrid viral envelopes, obtained by core-constitution of modified (bearing covalently attached insulin molecules) and unmodified viral glycoproteins, were highly hemolytic (Figure 4B).

The results in Figure 5 further support the view that virus-attached insulin molecules can mediate functional binding

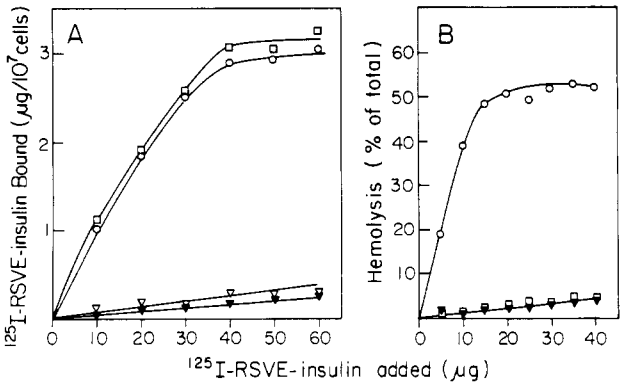

FIGURE 4: Ability of virus-attached insulin molecules to mediate functional binding of RSVE to desialized HE. All experimental conditions of attachment of insulin to ^{125}I-labeled Sendai virus envelopes and reconstitution of hemolytic viral envelopes bearing insulin molecules (unmodified to modified glycoproteins ratio in the detergent solution 3:1) were as described under Experimental Procedures and in Figure 1. Increasing concentrations of RSVE (▽), RSVE-bearing insulin molecules that were reconstructed in the presence of (○) or absence (□) of unmodified viral glycoproteins, or RSVE-bearing BSA that were reconstituted with unmodified viral glycoproteins (▼) were incubated with 10^7 desialized HE (200 μL of solution A). All other conditions of estimation of binding (A) to RSVE and the RSVE-induced hemolysis (B) of desialized HE were as described under Experimental Procedures and in Figure 1.

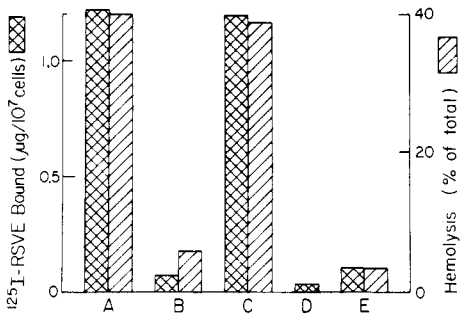

FIGURE 5: Effect of excess free insulin on binding and hemolytic activities of RSVE–insulin. Hemolytic ^{125}I-RSVE–insulins were obtained by mixing detergent solutions of unmodified and modified glycoproteins, as described in Figure 2 for RSVE-bearing antibodies. RSVE–insulin (A) or RSVE–BSA (E) (10 μg) were incubated with desialized HE. In systems B and C, 200 μg of free insulin or anti-HE-F(ab), respectively, was incubated wtih desialized HE (30 min at 20 °C) prior to the addition of the RSVE–insulin. In system D, 200 μg of insulin was added at the end of the incubation period (40 min at 20 °C) to a suspension containing the RSVE–insulin and desialized HE. The suspension was then allowed to incubate with the free insulin molecules for another 30 min at 20 °C. Binding of RSVE–insulin (cross-hatched) and RSVE–insulin-induced hemolysis (hatched) of desialized HE were as described in Figure 1.

of the virus envelopes to desialized HE. Free insulin molecules were able to avoid binding to the RSVE–insulin as well as RSVE-induced hemolysis, when added to desialized HE prior to the addition of ^{125}I-RSVE–insulin (Figure 5B). F(ab) molecules obtained from anti-HE-Ab that were able to avoid binding of viral envelopes, bearing anti-HE-Ab, to desialized HE (Figure 2) did not have any effect on the binding of RSVE–insulin (Figure 5C).

Discussion

The results of this work describe a novel method for the construction of highly fusogenic, targeted Sendai virus envelopes. Anti-HE-Ab were coupled to Sendai virus envelope glycoproteins dissolved in Triton X-100. Removal of the detergent led to the formation of RSVE bearing covalently bound antibody molecules. Such antibody molecules were able to mediate the binding of these vesicles to virus receptor depleted HE. However, these vesicles were unable to fuse with the HE membrane, as was inferred from their inability to cause hemolysis of desialized HE and from electorn microscopy observations (not shown). Only the insertion of untreated, active viral envelope glycoproteins to the RSVE bearing antibodies reactivated the virus' fusogenic activity, as was inferred from its ability to induce hemolysis (Maeda et al., 1977).

In accordance with previous observations (Miura et al., 1982; Heath et al., 1983; Gitman & Loyter, 1984), the present work shows that the viral HN glycoprotein, besides being the viral binding protein, also plays an active role in the virus fusion process. Virus vesicles containing attached anti-HE-Ab and an active viral fusion protein (F glycoprotein), although able to attach to neuraminidase-treated HE, fail to hemolyze them. Insertion of the active HN glycoprotein did not change the binding properties of such vesicles (which occurred due to the presence of the antibodies) but did restore their fusogenic activity.

In addition to IgG, also the polypeptide hormone insulin was coupled to the viral glycoproteins. The fact that insulin is a polypeptide of small molecular weight (6000), allowed us to study the question of which of the viral glycoproteins bind the insulin molecule. Our results clearly show that insulin molecules are attached equally to the two viral envelope glycoproteins. It is most interesting to note that the virus-attached insulin molecules, despite their being of low molecular weight, were able to mediate functional binding of the viral envelopes to desialized cells. To the best of our knowledge, our results show for the first time that the interaction between a virus-bound hormone molecule and its membrane receptor can substitute for the interaction between the virus binding protein and the cell receptor. The presence of insulin receptors in membranes of HE has been reported (Gambhir et al., 1978), and therefore, the cells could serve as a model system in our studies.

It may be speculated that the RSVE-bearing antibody or insulin molecules will serve as an efficient vehicle for the introduction of macromolecules into a selective cell population in culture and, eventually, in the whole animal. Indeed, preliminary results in our laboratory showed that fragment A of ricin and SV_{40} DNA can be enclosed within RSVE-bearing insulin molecules. Incubation of such loaded RSVE with cultured cells lacking virus receptors led to microinjection of the toxin or the SV_{40} DNA molecules into the cells' cytoplasm. It seems to us that for microinjection of macromolecules and transfer of membrane proteins (Prujansky-Jakobovitz et al., 1981) into cultured cells the use of RSVE may offer some advantages over the use of liposomes. RSVE fuse with cell plasma membranes, thus injecting their content directly into the cell cytoplasm or inserting their membrane components into recipient cell membranes. Liposomes, on the other hand, are taken into cells by endocytosis (Pagano & Weinstein, 1978), and in many cases, their use requires the addition of poly(ethylene glycol) (Doyle et al., 1979) or glycerol (Rizzo et al., 1983), both of which affect cell viability. However, loaded liposomes have been shown to be able to carry their content into tissues of whole animals (Chapman et al., 1984). Currently, such experiments are conducted in our laboratory with loaded RSVE. It will be extremely interesting to find out whether RSVE will be able to fuse with and inject their content into cells of specific tissues of whole animals.

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Resonance Raman Studies of the Flavin and Iron-Sulfur Centers of Milk Xanthine Oxidase†

Lawrence J. Willis‡ and Thomas M. Loehr*
Department of Chemical, Biological, and Environmental Sciences, Oregon Graduate Center, Beaverton, Oregon 97006
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ABSTRACT: Resonance Raman spectroscopy has been used to study milk xanthine oxidase, an enzyme containing molybdenum, binuclear iron-sulfur clusters, and FAD as cofactors. The contribution of FAD dominates the resonance Raman spectrum at frequencies above 500 cm^{-1}. As expected, no bands assignable to FAD are observed in deflavo xanthine oxidase. The resonance Raman spectrum below 500 cm^{-1} reveals the contribution of the $Fe_2S_2(Cys)_4$ groups with frequencies similar to those of adrenodoxin and putidaredoxin. Resonance enhancement profiles of the $Fe_2S_2(Cys)_4$ clusters indicate intensity variations among the $Fe_2S_2(Cys)_4$ peaks that are attributed to different excitation wavelength maxima of their bridging and terminal iron-sulfur vibrations. No evidence for Mo-ligand vibrations could be obtained by using excitation wavelengths between 363.8 and 514.5 nm.

Milk xanthine oxidase (EC 1.2.3.2) is a multicomponent molybdenum enzyme that catalyzes the two-electron oxidation of xanthine to uric acid (Spence, 1983). Important cofactors are two Mo(VI) centers, four $Fe_2S_2(Cys)_4$ groups, and two flavin adenine dinucleotide (FAD)[1] moieties per molecule of enzyme. Information about these cofactors has been obtained through the use of EPR, EXAFS, electrochemistry, and electronic absorption spectroscopy (Spence, 1983; Bray, 1980).

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‡ Present address: Department of Chemistry, Lewis & Clark College, Portland, OR 97219. Portions of this work were submitted by L.J.W. in partial fulfillment of the requirements for the Doctor of Philosophy degree awarded by the Oregon Graduate Center.

[1] Abbreviations: FAD, flavin adenine dinucleotide; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; CT, charge transfer; S_b and S_t, bridging and terminal sulfur, respectively; Cys, cysteine; XO, xanthine oxidase.

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