

Separation of Sendai Virus Glycoproteins by Using Glutaraldehyde-Treated Erythrocytes and Preparation of Monospecific Antisera Against the Glycoproteins

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Sendai virus hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins, F and HN, were separated from Triton X-100- or Nonidet P-40-solubilized envelopes as unadsorbed and eluted fractions, respectively, by using glutaraldehyde-treated chicken erythrocytes. These separated glycoproteins were biologically active. Monospecific antisera (in terms of monoreactivity to virus glycoproteins in gel diffusion precipitation patterns) were prepared by using these fractions as immunogens. Anti-HN rabbit serum inhibited all of the viral activities tested (infectivity, neuraminidase, hemagglutinating, and viral hemolysis), whereas anti-F serum definitely inhibited viral hemolysis only, although the two antisera enhanced neutralization in the presence of complement. The advantages and disadvantages of this separation method were discussed.

Various methods have been reported for the separation of Sendai virus and other paramyxovirus glycoproteins, such as fetuin (2, 10, 17) or lectin-Sepharose (5) affinity chromatography, zonal sedimentation (15, 16, 18), electrofocusing (14, 20), and DEAE-Bio-Gel A column chromatography (14, 22). A simple method for separation of hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins, the latter of which have no hemagglutination (HA) and neuraminidase activity, is to separate them by using erythrocytes. Since virus glycoproteins are dissociated only in the presence of detergent, detergent-resistant erythrocytes should be employed for this purpose. Glutaraldehyde (GA)-treated chicken erythrocytes have been used for the purification of a virus with hemolytic activity, Sendai virus, by Hosaka and Hosokawa (7).

The present report describes the separation of Sendai virus glycoproteins F and HN by using GA-treated erythrocytes and the characteristic inhibitions, or reactions, of the monospecific antisera prepared by using these fractions as immunogens.

MATERIALS AND METHODS

Virus. Sendai virus, Z strain, was used throughout the present experiments. Infectious Sendai virus was grown in the chorioallantoic cavity of developing eggs for 3 days and purified by velocity sedimentation in sucrose gradients as described previously (7), but without the step of banding on a sucrose cushion, since the omission of this step did not affect the polypeptide pattern of the virus obtained.

Infectivity assay. Hemadsorption focus-forming

units were counted on L929 cell sheets with guinea pig erythrocytes (7).

HA, HA inhibition, and hemolytic activity assays. Assays were carried out as described previously (4).

Neuraminidase assay. Samples of 50 μ l of viral preparations were mixed with 100 μ l of fetuin (10 mg/ml, Spiro method; GIBCO, N.Y.) in 0.01 M sodium acetate (pH 4.5) containing 0.05 M sodium chloride and incubated at 37°C for 30 min. All the reagents were used in half the volumes used in the original method of Aminoff (1).

Immunization of rabbits with virus antigens. Isolated virus antigens (200 and 150 μ g of protein for F and HN fractions, respectively) were injected intramuscularly, and 1 week later the same antigens were injected intravenously after they had been precipitated with cold butanol to remove detergent. The antigens were injected intramuscularly two or four times more, and 2 weeks after the last injection, the immunized rabbits were bled.

Antisera against virus particles were obtained from rabbits immunized with purified virions (10,000 hemagglutination units) injected intramuscularly and intravenously.

Immunodiffusion. The double diffusion technique was carried out in 1% agarose A-37 (Nakarai Chemicals, Kyoto, Japan).

Treatment of erythrocytes with GA. Treatment of chicken erythrocytes with GA was carried out as described previously (7), except that a higher concentration (1.0%) of GA was used.

Polyacrylamide gel electrophoresis. Disk electrophoresis was done by the method of Shimizu et al. (19).

Chemical determination. Protein was measured by the method of Lowry et al. (11).

Electron microscopy. Separated glycoprotein

fractions before and after precipitation with 10 volumes of cold butanol were examined by electron microscopy (Hitachi, type 12A) by using the negative staining technique with 2% phosphotungstic acid.

RESULTS

Separation of Sendai virus glycoproteins with GA-treated erythrocytes. Purified Sendai virions (16,000 HA units per ml) were solubilized with a high salt (1 M KCl) concentration plus 2% Triton X-100, and the solubilized preparation was freed from nucleocapsids and matrix proteins by centrifugation and dialysis (16). This

glycoprotein preparation was made isotonic by the addition of sodium chloride and then fractionated by the scheme shown in Fig. 1.

Table 1 shows the distributions of neuraminidase activity, HA, and protein in the resulting fractions. The unadsorbed fraction was almost neuraminidase-negative, but the eluted fractions had high neuraminidase and HA activities, the activities decreasing progressively upon repeated elutions. Sometimes, when old glycoprotein preparations were used, some neuraminidase activity remained in the unadsorbed fraction. In such cases, a protein of 59,000 daltons,

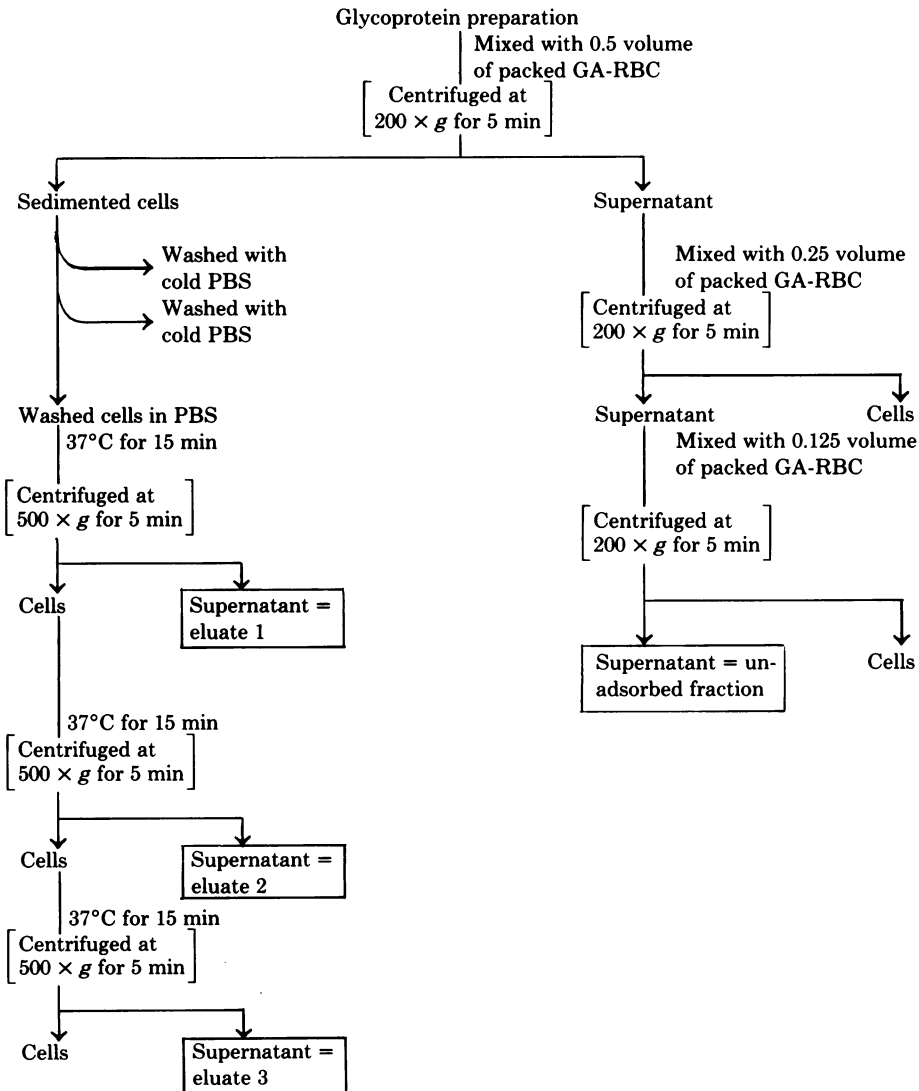


FIG. 1. Scheme of preparation of Sendai virus glycoproteins with GA-RBC. PBS, Phosphate-buffered saline.

TABLE 1. *Properties of fractions separated with GA-RBC from Sendai virus glycoprotein preparation*^a

Fraction	Neuraminidase activity (OD ₅₄₉) ^b	HA units per ml	Protein content (μg/ml)
Original preparation	1.85	ND ^c	230
Unadsorbed	0.01	ND	90
Washing			
1	0.75	120	ND
2	0.49	500	ND
Eluate			
1	1.20	10,000	68
2	0.63	1,200	40
3	0.42	1,000	30

^a A 15-ml portion of virus glycoprotein preparation was mixed with 7.5 ml of packed GA-RBC and then processed as described in the text. Volumes of washings and eluates were all 15 ml.

^b Samples of 10 μl of fraction were assayed. OD₅₄₉, Optical density at 549 nm.

^c ND, Not determined due to presence of Triton X-100.

which corresponded to hemagglutinin-free neuraminidase cleaved from HN protein (21), was detected by gel electrophoresis, in addition to HN and F (see Fig. 2). Therefore, fresh glycoprotein preparations were used.

Components of the fractions separated. Virus components in fractions separated by the GA-treated erythrocyte (GA-RBC) method were examined by gel electrophoresis, the gel diffusion technique, and electron microscopy.

Figure 2 shows the gel electrophoresis pattern of the glycoprotein preparation and of the unadsorbed and eluate 1 fractions, with reference to the virion pattern. The glycoprotein preparation showed two main bands, HN and F, whereas the unadsorbed fraction showed a main F band, with a small contamination of HN band, and the eluate 1 exhibited only HN band.

Figure 3 shows the gel diffusion patterns of the same three fractions. The glycoprotein preparation contains two components precipitating with anti-Sendai virus serum, and the unadsorbed and eluate 1 fractions each contain one antigenic component, each giving a line fusing with one of the two precipitation lines by the glycoprotein preparation, and crossing each other. These results indicate that these two components are antigenically different. Eluate 2 and 3 fractions were found to contain only HN protein by gel electrophoresis (data not shown).

Next, the unadsorbed and eluate 1 fractions

were examined by electron microscopy. Since the former fraction contained 2% Triton X-100, it was treated with cold butanol to remove the detergent and lipids. The precipitated protein consisted of aggregates of straight spikes of 14 nm in length, at the top of which there was a knob structure (Fig. 4A). The latter fraction was found to consist of fringed membranous structures. Since spike structures were not clearly resolved in this fraction, the fraction was similarly treated with butanol to remove lipids. The resulting precipitate consisted of aggregates of fibrous spikes of 11 to 12 nm in length (Fig. 4B). The morphologies of the spikes of these two fractions are consistent with those of HA-neuraminidase-positive and -negative spikes separated by electrofocusing of Sendai virus (20), and also with those of Simian virus 5 spikes separated by velocity sedimentation (15). The reason why particles in the eluate fraction were

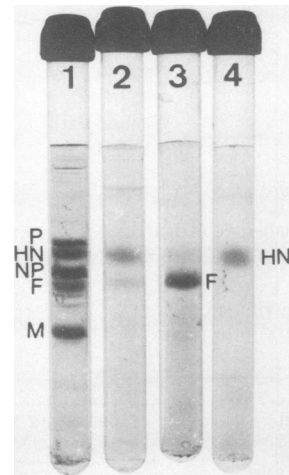


FIG. 2. *Gel electrophoresis patterns of Sendai virions (1), glycoprotein preparation (2), and unadsorbed (3) and eluate 1 (4) fractions. The protein bands were stained with Coomassie brilliant blue. Their designations are the same as those of Choppin and Compans (3).*

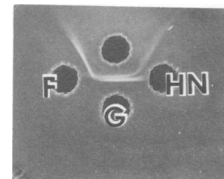


FIG. 3. *Gel diffusion pattern of the glycoprotein (G) and unadsorbed (F) and eluate 1 (HN) fractions against anti-whole Sendai virus serum in agarose gel. Eluate 1 fraction was treated with 0.25% Nonidet P-40 before application.*

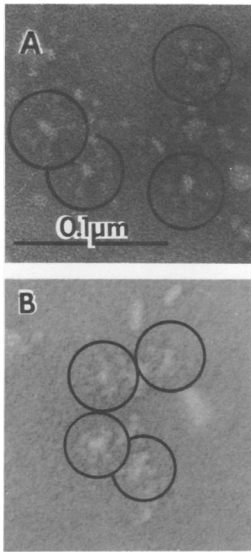


FIG. 4. Electron micrographs of the unadsorbed (A) and eluate 1 (B) fractions, precipitated with butanol. Negative staining was done with 2% phosphotungstic acid. Circles indicate aggregates of spikes.

associated with membranes was probably because HN proteins became associated with a small amount of lipids which remained there after the bulk of lipids and detergent was removed during the washing procedure. The above findings indicated that the unadsorbed and eluate 1 fractions are distinguished from each other in their polypeptide composition, antigenicity, and morphology.

Separation of Sendai virus glycoproteins from Nonidet P-40-solubilized envelopes by the GA-RBC method. Purified Sendai virions (16,000 HA units per ml) were treated with 0.25% Nonidet P-40 at room temperature for 15 min and then centrifuged at $100,000 \times g$ for 30 min (8), and the supernatant was treated by the GA-RBC method. In this case, the F and HN proteins were also found by gel electrophoresis to be almost confined to the unadsorbed and eluate fractions, respectively (data not shown).

Reassembly of particles with hemolytic activity from separated fractions. HN proteins in the eluate fraction were biologically active because they had HA and neuraminidase activities. The only way available to check the biological activity of isolated F proteins is to examine the reassembly of particles with hemolytic activity from the separated F protein fraction (8, 10). Table 2 shows that only reassembly of mixtures of unadsorbed and eluate fractions, which were separated from Nonidet P-40-solubilized envelopes, leads to formation of par-

ticles with hemolytic activity. Thus, F proteins in the unadsorbed fraction proved to be biologically active. The table also shows that the reassembled particles from the unadsorbed fraction alone had a slight HA titer. This is consistent with the finding that the fraction was contaminated with a slight amount of HN proteins, as shown by gel electrophoresis (Fig. 2).

Hemolysis-active particles were not reassembled from fractions that had been separated from Triton X-100-solubilized envelopes, probably because removal of Triton X-100 by the dialysis method employed was very slow.

Inhibitions of Sendai virus activities by monospecific antisera. Figure 5 shows the gel diffusion pattern of the antisera against the glycoprotein antigens prepared by using fractions separated by the GA-RBC method as immunogens. The pattern clearly demonstrates the monospecificity of these antisera in terms of reactivity against the virus glycoproteins.

Figure 6 shows the inhibitions of virus neuro-

TABLE 2. Reassembly of particles with hemolytic activity from separated fractions

Fractions or mixtures of fractions for reassembly ^a	Properties of particles reassembled ^b	
	HA (HAU/ml)	Hemolysis
Unadsorbed	100	0.018
Unadsorbed plus eluate 1	10,000	0.330
Eluate 1	20,000	0.005

^a Unadsorbed fraction (2.5 ml; 150 μ g of protein per ml) and 1 ml of eluate 1 (1 ml; 120 μ g of protein per 20,000 hemagglutination units per ml) were mixed or not mixed in the presence of Nonidet P-40 and dialyzed for 3 days through Spectrapor membrane no. 2 (Spectrum Medical Industries, Los Angeles) (9).

^b After dialysis, the preparations were centrifuged at $60,000 \times g$ for 30 min to remove remaining detergent, and the resulting pellets were suspended in 2 ml of phosphate-buffered saline for assay. HAU, Hemagglutination units. Hemolysis is reported in terms of OD₅₄₀ with 100- μ l samples.

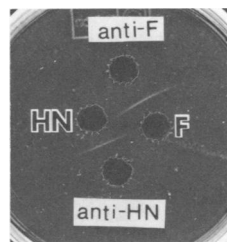


FIG. 5. Gel diffusion pattern of the unadsorbed (F) and eluate 1 (HN) fractions against monospecific antisera. The eluate 1 (HN) fraction was treated with Nonidet P-40 before application.

minidase activity and hemolysis with antisera against unadsorbed fractions (anti-F serum) and eluate fractions (anti-HN serum). Anti-HN serum efficiently inhibited both activities, but anti-F serum only inhibited hemolysis. The HA inhibition titer of anti-HN serum was 800, and that of anti-F serum was 80.

The low HA inhibition titer of the latter was probably at least in part due to contaminating anti-HN antibodies.

Figure 7 shows the curves for neutralization of Sendai virus by the two antisera. Anti-HN

serum strongly inhibited infectivity, but anti-F serum was inhibitory only at low dilutions. Neutralization by antiserum was enhanced in the presence of guinea pig complement (Table 3), indicating that complement-requiring neutralizing antibodies were present in both anti-F and anti-HN sera.

DISCUSSION

In this work, HN and F glycoproteins of Sendai virus were separated from each other by

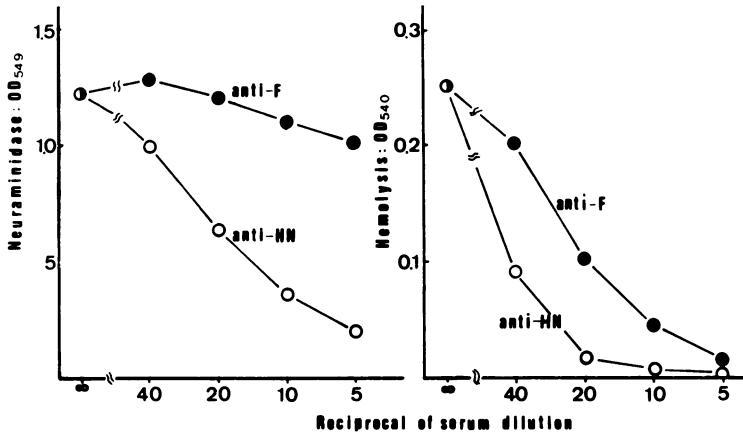


FIG. 6. Inhibition of Sendai virus neuraminidase activity (left panel), and hemolysis (right panel) with anti-HN and anti-F sera. Samples of 25- μ l of Sendai virus (100 HA units) were mixed with an equal volume of antiserum dilutions and the mixture was kept at room temperature for 60 min and then assayed for neuraminidase and hemolytic activities.

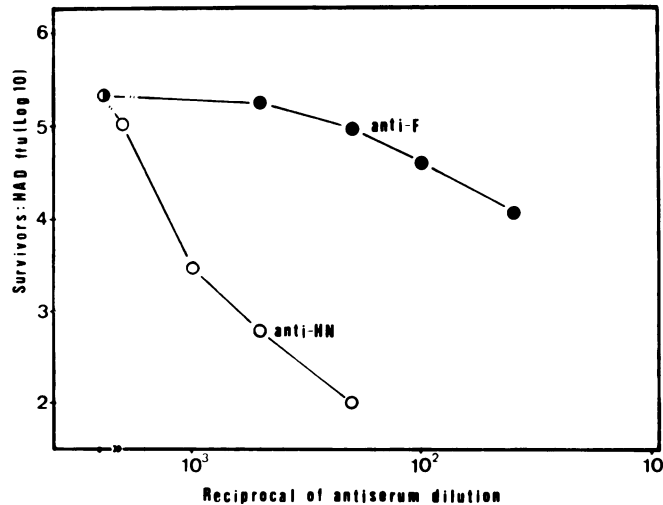


FIG. 7. Neutralization of Sendai virus infectivity with anti-HN and anti-F sera. Samples of 100 μ l of Sendai virus (1.9×10^6 hemadsorption focus-forming units) were treated with antisera similarly as described in Fig. 6. Then, the whole mixtures were inoculated onto L929 cell monolayers (Falcon 3001 plastic dish). After 24 h, the cells were covered with 2% guinea pig erythrocytes at room temperature for 1 h, and then extensively washed. Hemadsorption foci were counted under a light microscope.

TABLE 3. Enhancement of Sendai virus neutralization by anti-HN or anti-F serum with guinea pig complement

Antiserum	Hemadsorption focus number ^a at:				
	Guinea pig complement level (μ l) of				Heated complement (5.0 μ l) ^b
	0	1.0	2.0	5.0	
Anti-F (60-fold dilution)	2.69×10^4	8.32×10^3	2.64×10^3	5.6×10^2	2.90×10^4
Anti-HN (1,000-fold dilution)	2.32×10^3	ND ^c	80	ND	ND
Anti-HN (2,500-fold dilution)	1.80×10^5	ND	6.27×10^4	1.29×10^4	1.90×10^5
No antiserum ^d	1.90×10^5	1.88×10^5	1.90×10^5	1.60×10^5	ND

^a An amount of 1.9×10^5 hemadsorption focus-forming units per 100 μ l of Sendai virus was mixed with 100 μ l of antiserum dilution, and the mixture was kept at room temperature for 60 min and then incubated with complement at 37°C for 60 min. This mixture was inoculated on L929 cell sheets for hemadsorption focus assay. Values are averages of duplicate measurements.

^b Complement was heated at 56°C for 30 min.

^c ND, Not determined.

^d Instead of antiserum, 100 μ l of 2% fetal calf serum in phosphate-buffered saline was added.

using GA-RBC, and monospecific antisera were prepared by using these separated glycoproteins as immunogens. Since HN proteins were isolated by adsorption onto and elution from fixed erythrocytes, the proteins obtained had HA and neuraminidase activities. When a fresh glycoprotein preparation was used in which HN and F were completely separated and intact, a pure fraction of HN protein was obtained. The F fraction obtained was sometimes contaminated with a slight amount of HN proteins, which was detected as a very low HA titer when the unadsorbed fraction was dialyzed to remove detergent or as a faint HN band by gel electrophoresis. When an old glycoprotein preparation was used containing some free neuraminidase activity (a cleaved form of HN protein), this activity remained in the unadsorbed fraction. Further, if the glycoprotein preparation was contaminated with other viral proteins, they were recovered in the unadsorbed fraction. Therefore, this method is particularly useful for isolation of HN proteins with both HA and neuraminidase activity.

A disadvantage of this method was that sometimes the unadsorbed fraction became yellowish, probably because some hemoglobin or other material was released from the fixed erythrocytes. This contamination could be reduced by using fresh GA or a higher concentration of the reagent for fixation of cells.

All the monospecific antisera against HN and F glycoproteins obtained previously by different methods from paramyxoviruses had the following common characteristics: anti-HN serum inhibited all the viral activities tested, and anti-F serum inhibited viral hemolysis but not neuraminidase activity (2, 14, 18). The data of this study are consistent with these findings. Although some discrepancy has been observed in the extent of neutralization by anti-F serum,

depending on the serum, reported findings, with one exception (2), are consistent in indicating that anti-F serum has much lower neutralization activity than anti-HN serum (14, 18). This discrepancy can be explained by supposing that anti-F serum with neutralization activity is contaminated with various amounts of anti-HN antibodies. The non-HA inhibition hemolysis-inhibition antibodies described by Swedish investigators (12, 13) are thought to be anti-F antibodies.

ACKNOWLEDGMENTS

I thank K. Fukai for his support. The help of Y. Fukami and Y. Yasuda is also gratefully acknowledged.

This work was supported by a grant 337021 (1978, 1979) from the Ministry of Education, Science, and Culture of Japan.

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