Biochimica et Biophysica Acta 857 (1986) 1–12
Elsevier

BBA 73078

Preparation and characterization of F-protein vesicles isolated from Sendai virus by means of octyl glucoside

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(Received January 16th, 1986)

Key words: F protein; Glycoprotein; Membrane reconstitution; Membrane fusion; (Sendai virus)

We have demonstrated that Triton X-100 is always present in F-protein vesicles at concentrations that can provoke cell lysis. In order to avoid any misinterpretation of the fusogenic capacity of this protein, we solubilized the Sendai virus using octyl glucoside, which can be totally removed from the F protein preparation in less than 16 h by dialysis in the presence of absorbent beads. F-glycoprotein preparations preserved their ability to lyse erythrocytes in the presence of lectins and to induce cell-vesicle fusion as demonstrated by ESR studies. These vesicles were characterized by electron microscopy and SDS-polyacrylamide gel electrophoresis. Lipid analysis of these preparations by thin-layer chromatography indicated that they had the same proportion of lipids as virus envelopes, with slight variations in the sphingomyelin content and the cholesterol/phospholipid molar ratio. F-protein vesicles of different sizes can be obtained by adding exogenous lipids before detergent removal. The hemolytic activity of the vesicles was retained over a large range of lipid concentrations. We conclude that F-protein vesicles prepared with octyl glucoside are convenient tools for studying the fusogenic mechanism of this protein and improving the fusion process between liposomes and cells.

Introduction

The envelope of the Sendai virus contains two glycoproteins (HN and F) anchored in the lipid bilayer of the outer leaflet [1]. The functions of the two proteins have been extensively studied in relation to the infective process of the virus [2]. The F glycoprotein is required for viral entry into the host cells and for cell-cell fusion, and the HN glycoprotein causes adsorption of the virus to the cell surface and may also actively participate in the process of virus-cell fusion itself, as recently proposed by Gitman and Loyter [4]. Envelopes reconstituted after solubilization of intact virus were capable of agglutinating and lysing cells as well as fusing with their membranes [5,6]. Our objective was to separate the F protein in a form whose degree of purity would facilitate further studies of its fusogenic mechanism. Knowledge of this mechanism would be useful in improving the fusion process between liposomes and cells which is extensively studied in pharmacology and genetic engineering.

The use of a detergent to solubilize the viral envelope is a common feature of all techniques involved in F-protein isolation. Loyter and Volsky [3] recently reviewed the different methods used. Three main problems are encountered in the course of this preparation: sufficient separation of the two glycoproteins into their pure form, adequate

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

detergent removal, and reconstitution of functional membrane vesicles.

Tomasi and Loyter [7] have proposed an elegant method for obtaining large quantities of Sendai virus F glycoprotein which does not require the use of affinity chromatography [8]. This efficient and simple method involves a reduction of the disulfide bonds of the glycoproteins, prior to the addition of Triton X-100, which solubilizes only the F protein. The use of this detergent is very controversial. On the one hand, Triton X-100 is a nonionic detergent that preserves the functional activity of membrane proteins, but on the other hand, it has a low critical micellar concentration, which makes it difficult to remove by dialysis. Residual detergent can be of crucial importance when measuring the lytic activity of reconstituted vesicles with F protein, since the detergent leads to the formation of mixed micelles which modify cell-membrane integrity [9] and thus provoke lysis of red blood cells.

Results concerning Triton X-100 elimination are not very clear. Even with extensive dialysis, 10% residual detergent can be found in vesicles after many days [10,11]. The rate of dialysis and consequently the efficiency of detergent removal have been improved using Spectrapor tubing or Bio-Beads SM2. Nevertheless, we have computed values varying from 4% [10] to 0.25% [12] residual Triton X-100 in liposomes and 0.02% [3] to 0.2% [13] in reconstituted vesicles. Hsu et al. [8] even consider that Triton X-100 should no longer be used in F-protein isolation.

In this context, we sought a detergent capable of solubilizing membrane proteins without denaturing them and whose critical micellar concentration would be high enough to favor a quick and complete removal by dialysis. *n*-Octyl glucoside has a high CMC (24 mM) and has been successfully used to solubilize membrane proteins [14–16]. Its properties are now well defined [17] and we have demonstrated its complete removal from dialysate [12]. We here present results showing that reconstituted F-protein vesicles obtained with octyl glucoside preserve protein functions, and we analyze different characteristics of the vesicles as a function of the lipid/protein ratio.

Materials and Methods

Chemicals and isotopes. [^{3}H]Triton X-100 (1.58 mCi/mg); octyl β-D-[^{14}C]glucopyranoside (314 mCi/mmol) and [^{3}H]inulin (215 mCi/g) were purchased from New England Nuclear (France). di[1-^{14}C]palmitoylphosphatidylcholine (59 mCi/mmol) and iodine-125 were obtained from Amersham (France). Egg-yolk phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylserine from bovine brain and cholesterol were of the highest purity available from Sigma and were not further purified. Spin-labeled phospholipid (1–14 PC) was synthesized according to Hubbell and McConnell [18]. All chemicals used were of analytical grade. Dithiothreitol was provided by Sigma; *n*-octyl glucoside and lactoperoxidase by Boehringer-Mannheim and Triton X-100 by Merck. Lectins came from IBF (France); Bio-Beads SM2 and Bio-Gel A 1.5m from Bio-Rad (Richmond, U.S.A.).

Virus. Sendai virus (inoculum kindly provided by N. Kesler from the National Influenza Center, Lyon, France) was grown in 10-day-old embryonated chicken eggs (4–64 HAU/egg). The eggs were incubated for an additional 3 days after inoculation and the allantoic fluids were collected and clarified at $700 \times g$ for 10 min. Viruses were pelleted at $34\,000 \times g$ for 90 min, washed once with 10 mM Tris-HCl (pH 7.4)/150 mM NaCl (buffer A) and then resuspended in the same buffer. Each egg routinely provided about 6000 to 7000 HAU.

Hemagglutination titrations. Virus suspensions were assayed according to the technique of Salk and Pattern [33], using microtiter plates with 0.3 ml round-bottom cups: serial 2-fold dilution in buffer A and 50 μl of 1% human erythrocytes (outdated AB^+ red blood cells, washed with buffer A). Readings were taken after 60 min at 4°C. For the hemagglutination test of F-proptein preparations in the presence of lectins, wheat-germ agglutinin or phytohemagglutinin was added to the protein solution (3–30 μg/sample), which was further analyzed like the viral preparation.

F protein preparation. F protein was isolated according to Tomasi and Loyter's technqiue [7], which involves reduction of intact virus particles with dithiothreitol and solubilization of the

reduced virus with detergent. This process yields a solution containing the F protein as the main protein component after removal of all insoluble viral proteins by centrifugation. Detergent was subsequently removed by dialysis. We made minor changes in the technique such as a longer incubation of the virus with 3 mM dithiothreitol (12 h at 37°C), the use of octyl glucoside instead of Triton X-100, and the addition of Bio-Beads SM2 (9 g Bio-Beads per mmol detergent) to the dialysis medium. Trace amounts of radioactive detergent were added in some experiments to determine the residual detergent at the end of dialysis. Protein was determined as in Ref. 19.

Iodination of F protein. The protein was iodinated using the enzyme lactoperoxidase, according to Maeda et al. [20]. For this purpose, 500 μg of F-protein vesicles were placed in buffer A (pH 7.4), containing 1 mM EDTA, 30 μg lactoperoxidase, 2 nmol NaI, 50 μCi carrier-free $Na^{125}I$ at final volume of 1 ml. Hydrogen peroxide (5 μmol) was added to initiate the reaction at 0°C and an another 5 μmol of H_2O_2 was added after 5 min. 10 min later, the radioactive sample was dialyzed against buffer A and the purity of ^{125}I-labeled F protein was checked by gel electrophoresis. The specific radioactivity was about 100 cpm/ng protein.

Reconstitution and measure of entrapment. Variable amounts of an organic solution of mixed lipids (PC/PE/PS/SM/cholesterol) at a molar ratio of 1:2:1:1 [6] in some cases with trace amounts of [^{14}C]phosphatidylcholine, were evaporated in test tubes under a nitrogen stream, then dried in vacuo. An F-protein preparation containing ^{125}I-labeled F protein (100 cpm/ng protein) as marker was solubilized by octyl glucoside (detergent : protein = 4 : 1, w/w) and an identical aliquot was placed in each tube with [^{3}H]inulin as tracer ($(60–70) \cdot 10^6$ cpm/sample). F-protein vesicles were reconstituted after removal of the detergent by dialysis in the presence of SM2 Bio-Beads. Non-encapsulated inulin was washed from the vesicles by gel chromatography on a column (1.2 $\times$ 10 cm) of Bio-Gel A 1.5m equilibrated with buffer A. Encapsulation efficiencies (percentage of the original solute entrapped in the vesicles) were estimated from the tritium radioactivity of fractions. The internal volume of the vesicles was calculated on the basis of a surface area of 0.75 nm^2 per phospholipid molecule [21] with the following equation:

$$\% \text{ encapsulation} = 37 \cdot M \cdot d \cdot 10^2 \quad (1)$$

where M is the concentration of phospholipids (mol/liter) and d the diameter (μm) [12]. Phospholipid/protein molar ratios in reconstituted F-protein vesicles were calculated from the specific radioactivity of each component and specific determination of protein [19] and phospholipid [22] concentrations.

Hemolysis assay. Washed AB$^+$ erythrocytes were resuspended to a concentration of 1% (v/v) in buffer A, then 0.5 ml of the suspension was mixed with 0.25 ml of viral preparation or reconstituted F-protein preparation containing lectin, and kept for 15 min at 4°C before incubation for 25 min at 37°C. 2 ml of buffer A were added to each sample; then the mixture were spun at 700 $\times$ g for 5 min and the amount of hemoglobin in the supernatant was measured by its absorption at 540 nm. Identical assays in the absence of virus or F protein preparation but in the presence of lectin were used as controls.

ESR measurements. Spin-labeled F-protein vesicles (F-protein vesicles*) were prepared by solubilizing the ^{125}I-labeled F protein preparations (1 mg/ml) with octyl glucoside at the ratio 1 : 4 (w/w) then by coincubation with a dispersion of 1–14 PC, for 1 h at 20°C, to obtain a concentration of 1–14 PC in the F-protein vesicles* of 0.1 mM. F-protein vesicles were reconstituted by a 6 h dialysis and further purified from rosettes by centrifugation through a continous sucrose gradient (10–60%) at 100 000 $\times$ g for 24 h. The F-protein vesicle* fractions were pooled and subsequently washed twice by centrifugation at 100 000 $\times$ g for 2 h at 4°C.

The trypsin treatment of the F-protein vesicles* (30 μg) was carried out for 2 h at 37°C at a final enzyme concentration of 70 mg/ml. Vesicles were then washed twice with buffer A. 75% of the original proteins was found in the trypsinized fraction as evaluated by ^{125}I radioactivity.

In ESR measurements, F-protein vesicles* (3 μg) were added to 0.1 ml of 50% red blood cells in the presence of various amounts of wheat-germ agglutinin and left for 30 min at 0°C. Several

controls were carried out, first by removing wheat-germ agglutinin, then by replacing cells with water, ethanol or phosphatidylcholine liposomes (2 mM). The ESR spectra were measured at 37°C with a Brucker ER 200D spectrometer, equipped with a temperature-regulated cavity.

Polyacrylamide gel electrophoresis. The electrophoresis was performed on a separating gel containing 10% acrylamide and 0.28% bisacrylamide (sometimes with a linear gradient of 5–18% acrylamide) with 5% acrylamide as the stacking gel. Virus F-protein preparations were dissolved in 1% SDS/10% β-mercaptoethanol/500 mM Tris-HCl (pH 6.8)/30% glycerol and heated for 1 min at 100°C. Suitable samples (about 100 μg virus, 10–40 μg F protein) were subjected to electrophoresis at 10 mA for 16 h and stained with 0.25% Coomassie blue in 50% methanol and 5% acetic acid [23]. After electrophoresis, the slab gels were dried. In experiments using ^{125}I-labeled F protein, the gels were fractionated into 5-mm slices and counted in an ESI-Panax-counter.

Lipid analysis. Lipids were extracted from virus or F-protein preparations according to the method of Folch et al. [24]. Organic extracts were dried under nitrogen and the residues were dissolved in chloroform/methanol (2 : 1) for further analysis. Before lipid fractionation, aliquots were taken for measuring the total phospholipid content of the samples [22]. Lipid extracts were then fractionated by thin-layer chromatography on precoated silica-gel plates (Merck No. 5721), preactivated for 1 h at 100°C. The plates were developped in chloroform/ methanol/ acetic acid/ sodium bisulfite (50 : 20 : 6: : 1.5, v/v), which provided a better separation of phosphatidylserine and phosphatidic acid [25]. After visualization with iodine vapor, lipid spots were scraped and the phospholipids were directly measured on the gel.

Cholesterol was assayed as free cholesterol after saponification of organic extracts with 20% alcoholic potassium hydroxyde for 45 min at 90°C. The aqueous solutions were extracted with chloroform/water (5 : 1, v/v) and desiccated in the presence of sodium sulfate. Samples were trimethylsilylated with hexamethyldisilazane/ trimethylchlorosilane/pyridine (1 : 1 : 1, v/v) and left overnight at room temperature. Samples were appropriately diluted with hexane and gas-chromatographed on a Carlo Erba Fractovap 2300, with stigmasterol as standard.

Electron microscopy. Virus suspensions or F-protein vesicle preparations were applied to carbon-coated grids (300 mesh) and the excess liquid was blotted off before adding 0.1–1% uranyl acetate in water. The excess was again blotted off with filter paper and the grids were immediately examined in a JEOL JEM 200CR electron microscope at 100 kV.

The size distribution of the F-protein vesicles was determined by counting at least 200 vesicles in three separate experiments.

Results

F-protein preparation by octyl glucoside solubilization of virus

In order to improve the F-protein preparation from Sendai virus, we chose to use the technique of Tomasi and Loyter [7] with several changes (as described in Materials and Methods) because of its advantage of simplicity and efficiency. The reconstitution of F-protein vesicles with octyl glucoside was analyzed and the purity of the final sample was evaluated in comparison to triton X-100 preparation used by these authors. The experiments were performed in the presence of either octyl [^{14}C]glucoside or [^{3}H]Triton X-100, to quantify the detergent remaining after dialysis. The ratios of starting materials (detergent/viral proteins, w/w) needed to solubilize the viral suspensions were 4 and 2 for octyl glucoside and Triton X-100, respectively. In both cases, Bio-Beads SM2 were added to the dialysis medium at a ratio of 0.11 μmol detergent per mg beads, which provided the best elimination of both detergents [12], and were then changed several times.

Fig. 1 shows the results of removal of octyl glucoside and Triton X-100 by dialysis from a virus preparation. The octyl glucoside was almost completely removed from the medium in 15 h (less than 0.01% was associated with the pellet of F-protein-containing vesicles), whereas about 10% of the Triton X-100 remained in the medium (1% in the pellet) even after 120 h of dialysis in the presence of beads. Although direct contact between beads and detergent-solubilized virus provided sufficient detergent removal [26], this tech-

nique was avoided because more than 50% of the viral glycoproteins were lost, probably adsorbed onto the beads. We computed mean values for the detergent/phospholipid molar ratio in the vesicular preparation after dialysis, which amounted to less than 0.3 with octyl glucoside and about 15 with Triton X-100 (the detergent/protein molar ratio was less than 10 and about 180, respectively). The yield of F protein ranged from 4 to 6% with octyl glucoside, which was very similar to that obtained by Tomasi and Loyter [7] with Triton X-100.

Fig. 2 shows the polyacrylamide gel analysis of the proteins of native virus and F-protein preparations. In their reduced form, the F proteins are separated into their two polypeptide constituents, F1 and F2. No protein contaminant was found in the two preparations, indicating the relatively high purity of this protein, prepared with either detergent.

Fusogenic activity of F-protein preparation

The fusogenic power of an F-protein preparation is usually estimated by its capacity to hemolyze human erythrocytes in the presence of a lectin.

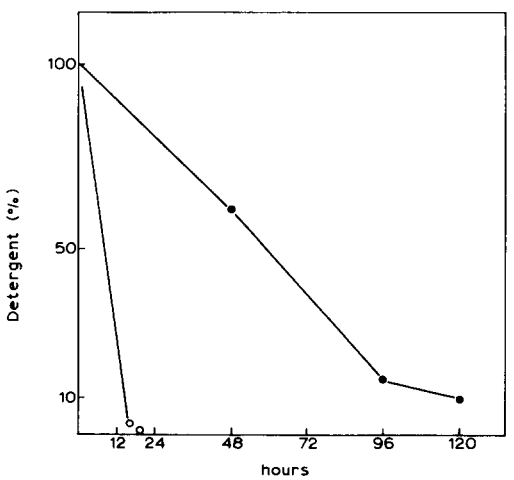

Fig. 1. Percentage of residual detergent in vesicle preparations dialyzed against buffer A (10 mM Tris-HCl (pH 7.4)/150 mM NaCl) containing Bio-Beads (9 mg beads/μmol detergent). Viruses were solubilized by octyl glucoside (○) or Triton X-100 (●) at a detergent/viral protein (w/w) ratio of 4 and 2, respectively. Trace amounts of [^{14}C]octyl glucoside or (^{3}H)Triton X-100 were added to samples before dialysis to check detergent removal. Aliquots of 10 μl were collected from the bags as a function of time and analyzed for radioactivity.

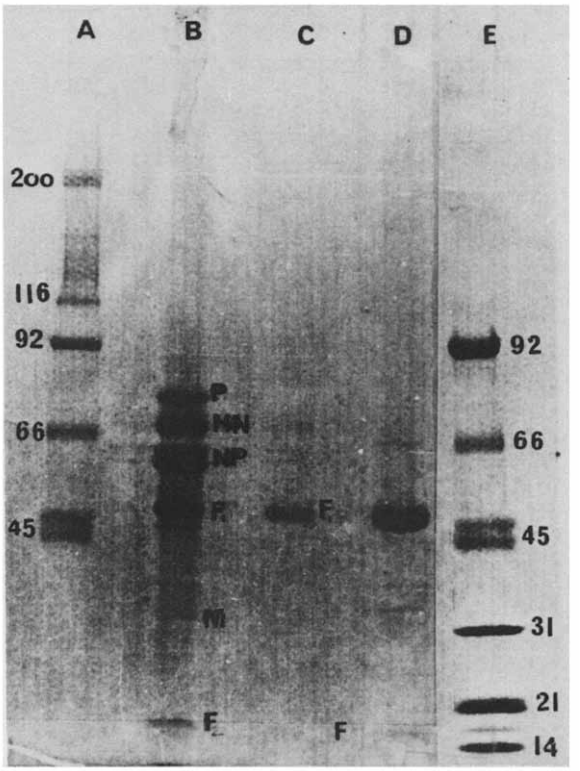

Fig. 2. SDS gel electrophoretic analysis of Sendai virus F glycoprotein in 5–18% (w/v) polyacrylamide gels: (A) and (E) are protein molecular weight standards; (B) 100 μg virus preparation in 1% SDS; (C) F protein prepared with Triton X-100, (D) F protein prepared with octyl glucoside, both loaded onto the gel in 1% SDS and 10% β-mercaptoethanol; (E). In (C) and (D) samples contained 10–40 μg proptein

Prior to any hemolytic test, we tested the ability of the vesicles to fuse with human erythrocytes by ESR measurement of the intermixing of lipids between vesicles and RBC membranes, in the presence of lectins [20]. The ESR spectrum of 1–14 PC, which was initially broadened in F-protein vesicles due to the strong spin-spin interaction between nitroxide radicals [27], was increased by the dilution of spin-labeled phospholipids in the red blood cell membranes after fusion. Fig. 3 shows the time-course of the spectral change of a mixture of F-protein vesicles* (labeled with 1–14 PC) and human erythrocytes in the presence of wheat-germ agglutinin. The relative ESR peak height increased rapidly with time and with the amount of wheat-germ agglutinin. It should be noted that labeled PC* exchanged even in the

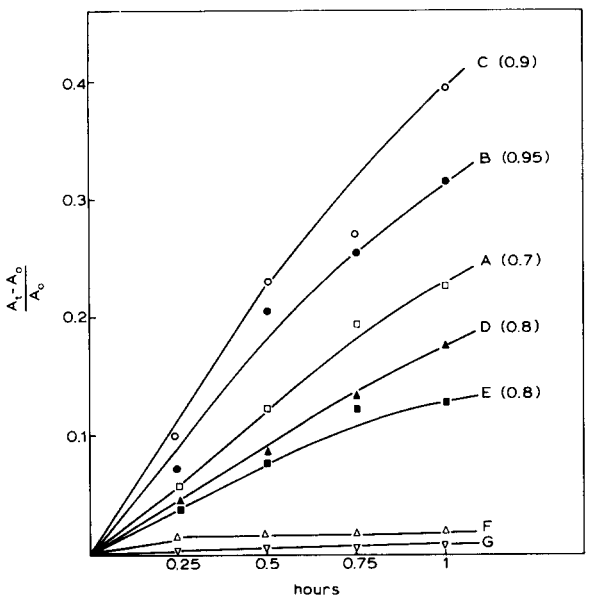

Fig. 3. Time-course of the spectral change of a mixture of F-protein vesicles* and human red blood cells at 37°C in presence of various concentrations of wheat-germ agglutinin. 3 μg F-protein vesicles, *, labeled with 1–14 PC, were incubated with 100 μl of a red blood cell suspension (50% hematocrit) without wheat-germ agglutinin (A) or in the presence of 10 μg (B) or 20 μg (C) of this lectin. The relative peak height was plotted against incubation times. In some experiments, F-protein vesicles* were trypsinized before incubation without (D) or with 20 μg of wheat-germ agglutinin (E). Controls were performed as follows. F-protein vesicles* were incubated with 20 μg wheat-germ agglutinin alone (F) or with phosphatidylcholine liposome (2 mM) plus 20 μg wheat-germ agglutinin (G). Figures in brackets correspond to data measured after 14 h.

absence of lectin (curve A), but that this exchange was nonspecific, since its magnitude was the same as that measured with trypsinized F-protein vesicles*, at whatever amount of wheat-germ agglutinin (curves D, E). On the other hand, the removal of cells (curve F) or their substitution by phosphatidylcholine liposomes (curve G) did not result in any spectral change. These data lead us to conclude that there was fusion between F-protein vesicles and red blood cell membranes. This fusion was specific in so far as it was function of: (i) the amount of wheat-germ agglutinin, (ii) the wholeness of the F protein (trypsinized protein was unreactive) and (iii) the presence of cell receptors (nothing appeared with liposomes).

It is possible to quantify this phenomenon with a fusion index, as follows:

$$\mathrm{FI} = \frac{A_t - A_0}{A_m - A_0} \times 100 \qquad (2)$$

where A_0 and A_t are the central peak heights at zero time and 14 h respectively, and A_m the central peak height when all labels are in red blood cell membranes. A_m was obtained from the same amount of (1–14) fatty acid used in the experiments, after their incorporation into the red blood cell membranes. All assays were performed at 37°C. FI (specific + nonspecific fusion) was equal to 35%, 51% and 45% in the presence of no 10 and 20 μg wheat-germ agglutinin, respectively. The data show that this FI (10 μg wheat-germ agglutinin) was 1.6-times higher than the FI (no wheat-germ agglutinin) of the intermixing lipid. We can estimate an FI corresponding to the specific fusion when A_0 becomes A_t with no wheat-germ agglutinin in Eqn. 2. These FI were equal to 28% and 11% for 10 and 20 μg wheat-germ agglutinin, respectively. We measured the fixation of the F-protein vesicle* (3 μg) to erythrocytes in 0.1 ml (50%, v/v) with 10 μg wheat-germ agglutinin. After 30 min at 4°C we found 20% of the F-protein vesicle* radioactivity bound to red blood cell membranes.

Lytic activity of F-protein preparations

Since F-protein vesicles can fuse with human erythrocytes in the presence of lectin, it is conceivable that the rate of hemolysis can be used to evaluate the efficacy of the F-protein preparation. The data in Table I show that, regardless of the detergent used, the F-protein preparations had a hemagglutinating activity in the presence of lectins, as well as a lytic power which increased with protein concentrations and remained indistinguishable from one detergent to the other. It should be noted that F-protein vesicles alone or wheat-germ agglutinin alone did not induce any significant hemolysis and similarly that trypsinized F-protein vesicles did not lyse erythrocytes in presence of wheat-germ agglutinin. Thus, the lysis observed was in fact a consequence of the fusogenic activity of F protein in the presence of wheat-germ agglutinin. Nevertheless, these activities were small compared to those of reconstituted fusogenic Sendai virus envelopes [25]. Further-

TABLE I

HEMOLYTIC ACTIVITIES OF RECONSTITUTED F-PROTEIN VESICLES

Hemagglutinating activity and lytic power of intact virus or F-protein vesicles prepared with octyl glucoside or Triton X-100, and assayed in the presence of lectins, were measured on AB^+ human erythrocytes at the concentrations stated below, as described in Materials and Methods. WGA, wheat-germ agglutinin; PHA, phytohemagglutinin.

System	Protein (μg)	HAU/mg	% hemolysis
Intact virus	3	10666	16
	5	12800	24
	10	12800	32
F protein	60	–	3
WGA (3 μg)	–	–	3
F protein isolated	36	56	10
with octylglucoside	50	40	13
+ WGA (3 μg)	60	33	14–15
	100	20	20
+ PHA (30 μg)	50	80	13
	60	67	17
Trypsinized F protein + WGA 3 μg	60	–	3
F-protein isolated with Triton X-100			
+ WGA (3 μg)	50	40	10
+ PHA (30 μg)	40	100	11

more, as noted by Osawa and Asano [6], a reconstituted system such as this needs a greater amount of F protein than native virus in order to produce the same degree of hemolysis. Hemolysis should increase with lectin concentrations, but lectins are toxic for cells and we did not use concentrations higher than 1 μM.

Since detergents are hemolytic agents, residual amounts of these compounds, namely Triton X-100, could have been responsible for part or all of the hemolysis observed when F-protein vesicles were in contact with red blood cells, in the presence of lectin. If this is the case, hemolysis cannot be used as an indicator of the F-protein activity. Thus, in order to clarify the hemolytic activity of F-protein vesicles prepared with each of the detergents, experiments were performed to evaluate the lytic power of these preparations due to residual detergent molecules. For this purpose, we compared the percent of hemolysis due to Triton X-100 alone to that provoked by F-protein vesicles (prepared with octylglucoside or Triton X-100) in the presence of the same amount of external Triton X-100.

Fig. 4 shows that Triton X-100 alone began to hemolyse erythrocytes at less than 0.04 mM (curve A) and was maximum at 0.16 mM. At the same concentrations of Triton X-100, in the presence of lectin and F-protein vesicles prepared with octyl glucoside, the hemolysis stayed almost invariable at low Triton X-100 concentrations (0–0.08 mM), and then suddenly rose and paralleled hemolysis by the detergent alone at high concentrations. Since there were no residual octyl glucoside molecules in these vesicles, these results suggest that Triton X-100 first balances with the vesicles (curve B1 is to the right of curve A), and then provokes hemolysis of the erythrocytes, as in the case of detergent alone at higher concentrations. The F-protein vesicles inactivated by trypsinization (curve B2) indicate the influence of Triton X-100 on hemolysis in the presence of non-hemolytic

vesicles. We observe that Triton X-100 began to hemolyse around 0.08 mM. By contrast, F-protein vesicles prepared with Triton X-100 contained, in this experiment, about 0.8% residual detergent after 4 days dialysis. Under our experimental conditions this amount corresponded to 0.1 mM Triton X-100, which is sufficient to provoke hemolysis of cells by desorption of the detergent from the vesicles into the medium (CMC = 0.24 mM). Thus, curve C (Fig. 4) was drawn to account for this residual detergent. The addition of external Triton X-100 to these vesicles did in fact immediately induce cell lysis (curve C), which parallels hemolysis by the other kind of vesicles and demonstrates the presence of residual detergent molecules in them. Thus, the hemolysis by a Triton X-100 preparation of F protein depends on F protein alone at Triton X-100 concentrations lower than 0.08 mM and on both F protein and residual detergent at higher concentrations of Triton X-100. Hence, data obtained with Triton X-100 preparations by F protein cannot be considered to be completely reliable and inevitably introduce a misinterpretation of the fusogenic capacity of these vesicles. Moreover, it is tedious and somewhat difficult to estimate the residual detergent concentration accurately. In our studies we still detected 0.2 mM Triton X-100, final concentration in some preparations for the hemolysis assay, which falsifies the results, since this level of concentration hides the specific effect of the F protein.

Lipid composition of F-protein vesicles

It is not well established whether the biological activity of the viral F protein depends on the presence of phospholipids. Only a few data are available on the lipid content of virus membranes and F-protein vesicles [28]. Table II lists the results obtained.

As shown by Blough and Lawson [28] the lipid content of the virus envelope is characterized by high levels of phosphatidylethanolamine and phosphatidylserine and a very low level of phosphatidylcholine, compared to the plasma membrane of eukaryotic cells [29,30]. However, our data perceptibly differ from previously published results [28], since we measured a very low concentration of phosphatidic acid and a higher phosphatidylcholine and sphingomyelin content, probably due to differences in the solvents and/or techniques used. It should be noted that the cholesterol/phospholipid molar ratio observed was very close to those in previous works on the

TABLE II

COMPARISON OF WHOLE LIPID CONTENT AND VARIOUS CLASSES OF PHOSPHOLIPIDS IN INTACT VIRUS AND F-PROTEIN PREPARATIONS

System	Phospholipid/protein [a] (nmol/mg)	Cholesterol/protein [a] (nmol/mg)	Cholesterol/phospholipid (mol/mol)	Phospholipid composition (% of total sample phospholipid)				
				PA	PE	PS	PC	SM
Intact virus	171 (7)	135 (3)	0.8	4	28–38	14–20	18–20	25–30
F protein prepared with octyl glucoside	1300 (3)	2700 (3)	2.0	4	30–40	16–22	20–25	20–25
F protein prepared with Triton X-100	1150 (3)	1900 (3)	1.65	5	25–35	18–26	25–40	5–10
Eukaryotic cells (see Refs. 29, 30)	–	–	–	2	15–25	5–45	45–55	5–25

[a] Per mg of total protein for virus and mg of F protein for vesicles. Figures in brackets correspond to the number of experiments.

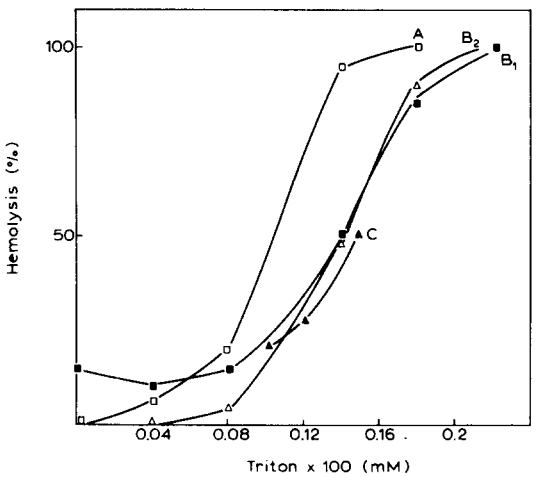

Fig. 4. Effects of Triton X-100 on the hemolytic activity of F-protein vesicles. A 1% red blood cell suspension was mixed with Triton X-100 solution at the indicated concentrations (A) (□) or with a reconstituted F-protein preparation containing wheat-germ agglutinin (3 μg) prior to detergent addition (B, C), then the hemolytic assay was continued as in Materials and Methods. There was 65 μg of F-protein per sample in a total volume of 1 ml. (B1) ■ corresponds to F protein vesicles prepared with octyl glucoside; (B2) △ to the same vesicles after trypsinization and (C) ▲ to the vesicles prepared with Triton X-100. In this last preparation we estimated the residual Triton X-100 concentration to 0.1 mM, which was counted with the external Triton X-100 addition. Data were corrected for the blanks. The hemolysis measured in the absence of detergent corresponds to the lytic activity of both F-protein preparations.

Sendai virus [3,30] and on eukaryotic plasma membranes [29,30].

The analysis of the lipid content in the two different preparations of F protein reveals several interesting details. F-protein vesicles prepared with octyl glucoside had proportionately the same lipid composition as virus envelopes. On the other hand, vesicles prepared with Triton X-100 differed with respect to their small amount of sphingomyelin. Furthermore, the data in Table II (columns 2 and 3) show that the isolation of F-protein vesicles corresponded to a relative lipid enrichment of these vesicles because of the elimination of other viral proteins. Nevertheless, this process involved a loss of lipid material (which probably remained bound to protein HN) as demonstrated by the rise in the cholesterol/phospholipid molar ratio. In this context, octyl glucoside preserved comparatively more cholesterol than Triton X-100 (same results as in Ref. 3).

Characteristics of F-protein vesicles as a function of their lipid content

In the course of vesicle formation by detergent removal it is possible to fashion the lipid composition, and thus change the size of the vesicles, and entrap macromolecules in the internal volume as, for example, in the case of drug encapsulation inside liposomes [31]. Table III lists the data of one experiment on F-protein vesicle reconstitution in the presence of different amounts of a lipid

TABLE III

CHARACTERISTICS OF VESICLES AS A FUNCTION OF THE LIPID:PROTEIN RATIO

The reconstitution of F-protein vesicles was carried out with octyl glucoside, as described in Materials and Methods, in the presence of radioactive inulin and variable amounts of exogenous lipids (PC, PE, PS, SM, Chol) in the ratio 1:2:1:1:5. The protein concentration was 0.5 μM in all samples. n.d., not determined.

Properties	Phospholipid to protein ratio (mol/mol) [a]			
	35 [b]	59	75	417
Phospholipid concn. (μM)	18	30	38.6	213
Insulin entrapped (%)	3.25	5.5	8.3	11
Diameter measured by electron microscopy (nm) [c]	40–50 (80)	n.d.	60–90 (70)	100–150 (70)
Internal volume (l/mol PL)	1.5–1.9	n.d.	3.0	5.0

[a] Measured after dialysis and gel filtration on Bio-Gel A 1.5m.

[b] Sample without exogenous lipid addition.

[c] Figures in brackets are mean percentage of vesicle diameters included in the range.

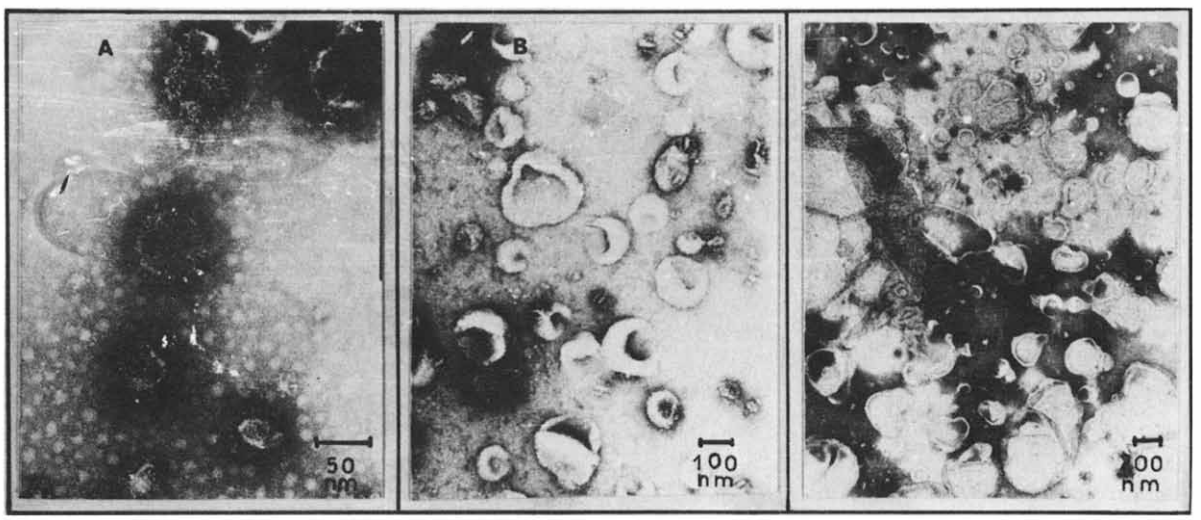

Fig. 5. Electron micrographs of negatively stained F-protein vesicles. These vesicles are the same as those described in Table III. (A) Vesicles formed without lipid added, and with a phospholipid/protein molar ratio equal to 35; (B) vesicles with a ratio of 75; (C) vesicles with a ratio of 417.

mixture containing PC/PE/PS/SM/cholesterol at a molar ratio of 1 : 2 : 11 : 1 : 5 [6]. Note that the increase in the native lipid content of vesicles as a result of loading the dialysis bag with exogenous lipids paralleled the increases in the percentage of entrapped material and the diameter of vesicles. These increases varied proportionally as long as the increase in the lipid/protein ratio was less than 2.2-fold. Fig. 5 shows an electron micrograph of F-protein vesicles prepared by means of octyl glucoside. The vesicles have an approximate diameter of between 40 and 100 nm. Spikes of F-protein are clearly visible, arranged in a rosette-like structure or sometimes in a fish-bone structure (5% of total protein). Regardless of the lipid/protein ratio, the vesicles have a unilamellar appearance (Figs. 4B and 4C), which is confirmed by comparing the internal volume calculated from Eqn. 1 with the estimated volume of the unilamellar vesicles in the same preparation, based on electron microscopic measurements.

Assays were performed to check the lytic activity of these different lipid-enriched F-protein vesicles. We verified that an increase in the lipid/protein ratio slightly modified the hemolytic power of the vesicles (shown in Table I). In a typical experiment (70 μg F protein, 3 μg wheat-germ agglutinin), the hemolysis of human red blood cells varied from 20% to 17% and 14%, whereas the lipid/protein ratio rose from 105 (no addition of exogenous lipids) to 305 and 485 (M/M), respectively. Thus, the largest vesicles were still hemolytically active.

Discussion

Our objective was to isolate the pure form of the F protein from the Sendai virus, i.e., distinct from other viral proteins, mainly the HN protein of the virus envelope, while removing any detergent residues and preserving its functional capacity to hemolyze and fuse cells. Fig. 4 shows that Triton X-100 can be involved in cell lysis because of residual detergent remaining associated with F-protein vesicles. Moreover, the removal of Triton X-100 by dialysis is a long process (Fig. 1) unless Bio-Beads are directly in contact with the detergent [25], but this technique leads to more than 50% F-protein loss. The use of octyl glucoside instead of Triton X-100 greatly improves F-protein preparation. No detergent was detected after 16 h of dialysis against buffer containing beads at a defined detergent/bead ratio (Fig. 1). The protein prepared in this manner was electrophoretically pure (Fig. 2) and preserved its capacity to fuse to human erythrocytes (Fig. 3) and to

lyse red blood cells (Table I). Over a large range of concentrations, exogenous lipids could be incorporated into F-protein vesicles without any appreciable decrease in lytic activity. The size and internal volume of these vesicles increased with the lipid content (Table III).

We demonstrate here that the fusion and lysis processes are directly related to the presence of lipid vesicles containing the F protein of the Sendai virus, excluding any other membrane protein. In our experiments the rate of these phenomena were weak compared to the same properties observed on whole virus (Table I) [20], or HN + F-containing vesicles [5]. Nevertheless it should be noted that two factors can also influence both of the previous phenomena, irrespective of the specific interaction between F protein and the target membrane.

First of all, the fusion process, as measured by ESR, assumes that there is a close contact between red blood cells and vesicles, via a wheat-germ agglutinin lectin. The proportion of vesicles in close contact with red blood cell membranes is hard to determine precisely because lectins provoke both cell-vesicle and cell-cell aggregations that cannot be computed. Nevertheless, the fusion process induced by F protein vesicles that was observed in our experiments (Table I) was far from being as negligible as a first observation would indicate. The percent of vesicles bound to red blood cells, one of the limiting factors of the fusion process, was low in our experiments (20% but the corresponding FI (specific fusion) was 28%.

On the other hand, hemolysis results from an increased fragility of red blood cell membranes as large molecules (hemoglobin) cross them. Although this leak is relevant to the fusion between vesicles and red blood cell membranes, it is difficult to determine what is the first cause of the hemolysis. Apart from the possibility of osmotic shock, excluded here, the increased fragility of the red blood cell membranes, and even their disruption, can be explained only by the dissociation of the membrane structure either by an effect of lipids (detergent or lipasic effects of F protein) or an effect of the membrane proteins, i.e., aggregation of intrinsic or extrinsic membrane proteins implying disruption. To date, none these phenomena has been shown to be relevant to F-protein vesicles, although some of them have been studied in the viral envelope [32]. The quantitative relation between fusion and hemolysis remains to be determined precisely with respect to yield and kinetics. Under these circumstances it would be hasty to draw conclusions from fusion and hemolysis data in order to characterize the role of the F protein in the mechanism of hemolysis.

In conclusion, the F-protein vesicles prepared by our technique exhibit the properties required for further studies of the fusion mechanism induced by this protein, and exclude any possibly misinterpretation of the observed phenomena. These vesicles also seem well-adapted to carrying materials such as drugs or macromolecules of biological interest, and facilitating their internalization by fusion once the vesicles are bound to cells by a targeting process (antibodies, specific ligands, lectins, etc.).

Acknowledgement

This work was financed by grnats from PIRMED (ASP No. 48), la Fondation pour la Recherche Médicale and l'Association pour la recherche sur le Cancer.

References

1 Choppin, P.W. and Compans, R.W. (1975) in Comprehensive Virology (Fraenkel-Conrat, H. and Wagner, R.R., eds.), Vol. 4, pp. 95–178, Plenum, New York
2 Rott, R. and Klenk, H.D. (1977) in Cell Surface Reviews (Poste, G. and Nicholson, G.L., eds.), Vol. 2, pp. 47–81, North-Holland, Amsterdam
3 Loyter, A. and Volsky, D.J. (1982) in Cell Surface Reviews (Poste, G. and Nicholson, G.L., eds.), Vol. 8, pp. 215–266, Elsevier Biomedical Press, Amsterdam
4 Gitman, A.G. and Loyter, A. (1984) J. Biol. Chem. 259, 9813–9820
5 Volsky, D.J. and Loyter, A. (1978) FEBS Lett. 92, 190–194
6 Osawa, M. and Asano, A. (1981) J. Biol. Chem. 256, 5954–5956
7 Tomasi, M. and Loyter, A. (1981) FEBS Lett. 131, 381–385
8 Hsu, M.C., Scheid, A. and Choppin, P.W. (1979) Virology 95, 476–491
9 Helenius, A. (1973) Ph.D. Thesis, Helsinki
10 Allen, T.M., Romans, A.Y., Kercret, H. and Segrest, J.P. (1980) Biochim. Biophys. Acta 601, 328–342
11 Scheule, R.K. and Gaffney, B.J. (1981) Anal. Biochem. 117, 61–66

12 Philippot, J.R., Mutaftschiev, S. and Liautard, J.P. (1983) Biochim. Biophys. Acta 734, 137–143
13 Kruse, C.A., Wisnieski, B.J. and Popjak, G. (1984) Biochim. Biophys. Acta 797, 40–50
14 Petri, W.A. and Wagner, R.R. (1979) J. Biol. Chem. 254, 4313–4316
15 Helenius, A., Fries, E. and Kartenbeck, J. (1977) J. Cell. Biol. 75, 866–880
16 Stubbs, G.W. and Litman, B.J. (1978) Biochemistry 17, 215–219
17 Rosevear, P., Vanaken, T., Baxter, J. and Ferguson-Miller, S. (1980) Biochemistry 19, 4108–4115
18 Hubbell, M.L., Mc Connell, H.M. (1971) J. Am. Chem. Soc. 93, 314–326
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
20 Maeda, T., Asano, A., Okada, Y. and Ohnishi, S. (1977) J. Virol. 21, 232–241
21 Huang, C. and Mason, J.T. (1978) Proc. Natl. Acad. Sci. USA 75, 308–310
22 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
23 Laemmli, U.K. (1970) Nature 227, 680–685
24 Folch, J., Lees, M. and Sloane Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509
25 Pullarkat, R.K., Sbaschnig-Agler, M. and Reha, H. (1981) Biochim. Biophys. Acta 663, 117–123
26 Vainstein, A., Hershkovitz, M., Israel, S., Rabin, S. and Loyter, A. (1984) Biochim. Biophys. Acta 773, 181–188
27 Scandella, C.J., Devaux, P.F. and McConnell, H.M. (1972) Proc. Natl. Acad. Sci USA 75, 4519–4523
28 Blough, H.A. and Lawson, D.E.M. (1968) Virology 36, 286–292
29 Wallach, D.F.H. (1975) Membrane Molecular Biology of Neoplastic Cells, pp. 141–181, Elsevier, Amsterdam
30 Van Deenen, L.L.M. and De Gier, J. (1975) in The Red Blood Cells (Surgenor, D., ed.), pp. 243–307, Academic Press, New York
31 Gregoriadis, G. (1984) Liposome Technology, Vol. 2. Incorporation of Drugs, Proteins and Genetic Materials, CRC Press, Boca Raton
32 Israel, S., Ginsberg, D., Laster, Y., Zakai, N., Milner, Y. and Loyter, A. (1982) Biochim. Biophys. Acta 732, 337–346
33 Salk, J.E. (1944) J. Immunol. 49, 87–88