MECHANISM OF MEMBRANE FUSION BY HVJ GLYCOPROTEINS

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CONTENT

GENERAL INTRODUCTION

HVJ (hemagglutinating virus of Japan, Sendai virus) is a virus belonging to the paramyxovirus group, and is famous for causing cell fusion (34). This virus is made up of three parts; the envelope consists of a lipid bilayer and two glycoproteins (F and HN), the nucleocapsid consists of RNA and two non-glycosylated proteins (NP and P) and matrix consists of M protein which is thought to line the envelope. The envelope is known to be important for induction of cell fusion (34). HVJ has been found to be a very useful tool for making hybrid cells in somatic cell genetics, and also has been used in the developement of new techniques to change the characteristics of mammalian cells artificially (ex. cell reconstitution method (8, 46, 55), ghost fusion method (9, 25, 43) and microinjection of macromolecules by liposomes associated with HVJ envelope glycoproteins (51)).

Cell fusion occurs spontaneously only in exceptional cases such as fertilization and formation of myotubes, and does not occur between normal cells under normal conditions. But when we change our eyes to the phenomenon at a single cell level, partial fusion of the cell membrane often occurs in normal cells (ex. endocytosis and exocytosis). Thus, the control of cell membrane dynamics is thought to be one of the most important event for the survival of cells. As the cell membrane is not a simple lipid bilayer, but a very complicated system containing proteins known as cell cytoskeleton, the investigation of cell membrane dynamics has faced many difficulties, although many efforts had been made. Thus analysis of changes in cell membranes during cell fusion will give insights for improvement of techniques in cell biology and for investigation of cell membrane dynamics.

HVJ has an envelope consisting of a lipid bylayer and two glycoproteins (F and HN), which is known to have an important role in cell fusion and viral

infection, allowing penetration of viral RNA and other viral components by fusion of virus envelope and cell membrane (34). One important problem which had concerned many people studying cell fusion was whether cell-cell fusion (fusion between cell membranes) resulted directly from envelope fusion (fusion of viral envelope and cell membrane). This problem was resolved clearly by the experiments using cytochalasin D (31). Cytochalasin D is a fungal metabolite, and is known to inhibit cell movement, such as cytokinesis, membrane ruffing, cell locomotion, phagocytosis, and capping of lymphocytes mediated by antiserum or lectins. Many of these drug effects seem to be caused by reversible disruption of microfilaments associated with the cell membrane, as this drug was known to be an inhibitor of myosin ATPase. Miyake et al. (31) showed this drug inhibits cell-cell fusion, but does not inhibit envelope fusion. Changes in the cell membrane necessary for cell-cell fusion seem to be caused by the changes in cytoskeleton proteins follow envelope fusion.

For this reason, it seems important to use "living cells" in experiments for the study of cell fusion phenomenon. During the long history of investigation of the cell fusion mechanism, human erythrocytes were the most popular materials for experiments. Although the erythrocyte membrane is easier to analyze than normal cell membranes because it is relatively simple in construction and is usually kept stationary state, it differs from normal cell membrane in many ways with regard to the conditions needed for cell fusion. For example, during the process of cell fusion, the erythrocyte does not need calcium ions and adenosine triphosphate (ATP) as an energy source, which other cells require (34). It also has no microfilaments, targets of cytochalasin D.

In this manuscript, we analyze virus envelope-cell membrane fusion and fusion between cell membranes using the simplest system consist of living cells and liposomes containing fragment A of diphtheria toxin trapped inside. Fragment A

contains the N terminal 21,150 sequence (4), and the NAD:elongation factor 2 (EF2) ADP ribosyl (ADPR) transferase activity of diphtheria toxin (10, 16). It blocks protein synthesis in eukaryotic cell extracts, but is not toxic to susceptible animals or cultured cells, because it cannot reach the cell cytoplasm (50). Thus, the toxicity to mammalian cells of lipid vesicles containing fragment A (i.e., activity for introduction of molecules from the lipid vesicles into the cell cytoplasm as the result of fusion between the cell membrane and vesicle membrane) indicates the efficiency of fusion between liposome and cell membrane.

This thesis consists of the following three parts.

Part 1: Analysis of fusion of viral envelope and cell membrane using liposomes reconstituted with purified virus envelope glycoproteins.

Part 2: Analysis of changes in membrane of normal cells caused by HVJ using liposomes.

Part 3: Analysis of fusion of liposomes and SSPE cell (subacute sclerosing panencephalitis virus-infected cell) membranes.

MATERIALS AND METHODS

Materials

Egg yolk lecithin (type V-E) and cholesterol (Grade1, 98%) were obtained from Sigma Chemical Co. (St Louis, Mo). DE-52 and CM-52 were obtained from Whatman Ltd. Nonidet P40 was obtained from Nakarai Chemicals Ltd. Neuraminidase from Cl. perfringens was obtained from Worthington Co. Ltd. Bio-Gel A50m, Bio-Gel A150m and Bio-Beads SM-2 were obtained from Bio-Rad Laboratories. [^{14}C]NAD (260 mCi/mmol) was obtained from New England Nuclear. Pristan (2,6,10,14-tetramethylpentadecane) was obtained from Aldlich Chemical Co.

Virus

HVJ, Z strain, was propergated in the chorioallantoic fluid of chick embryos. The virus was purified by differential centrifugation, as described previously (37).

The SSPE virus was the Biken strain isolated from brain cells of a patient by co-culture with human embryonic lung (HEL) cells (53). As no infectious virus is released into the culture medium from the SSPE-infected cells, the strain can only be passaged by cell-to-cell infection, with fresh cells added at each passage.

Cells and cell culture

HEL cells and CV-1 cells were used for culture and assay of SSPE virus. These cells were grown in monolayers in a mixture of equal volumes of Medium 199 and Eagle's minimal essential medium (199/MEM) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Mouse L cells were grown in monolayers in MEM supplemented 10% (v/v) bovine serum.

Neuraminidase assay and hemagglutination titration

Neuraminidase was assayed by the method of Aminoff (1) by measuring the

amount of reaction product formed. The reaction mixture contained 10 μg of N-acetyl-neuraminic lactose and 50 μl of test sample in 50 mM phosphate buffer (pH6.0) and the incubation was carried out at 37°C for 30 min.

Hemagglutination titration was carried out according to the method of Salk (40).

Hemolysis assay

Hemolytic activity was measured as described previously (35). A mixture of 0.15 ml of lipid vesicles (OD_{540} =0.075) and 0.3 ml of 2% chicken red blood cell suspension in SSC (150 mM NaCl, 15 mM sodium citrate) was kept cold for 5 min and then incubated at 37 C for 60 min. Then the mixture was centrifugated at 2000 rpm (400g) for 10 min and the amount of hemoglobin liberated into the supernatant was determined by measuring A_{540}. A reaction mixture containing 0.5% NP40, was used as a control to determine 100% hemolysis.

Purification of glycoproteins of HVJ

Purification of glycoproteins was performed by a slight modification of the method described previously (60).

Purified virions (10^6HAU) were suspended in 10 mM Tris-HCl (pH7.6) containing 0.2 M NaCl and 0.5 % NP40 to solubilize glycoproteins, and then centrifuged at 15000 rpm (20000g) for 60 min. The supernatant was dialysed against 10 mM acetate buffer (pH5.2), and the dialysed solution was centrifuged at 30000 rpm (80000g) for 30 min to remove insoluble materials. The supernatant, containing only F and HN (Fig.1, lane1), was applied to a column of CM-52 equilibrated with 10 mM acetate buffer (pH5.2) with 0.1% NP40. The flow through fraction contained pure F protein. HN protein was recovered by elution with 0.5 M NaCl. Fractions eluted with 0.2 M NaCl contained mainly F protein with small amounts of other proteins and a little HN protein. For further purification of HN protein, the material in fractions eluted with 0.5 M NaCl was dialysed against 5

mM phosphate buffer (pH6.0) and then applied to a column of DE-52 equilibrated with 5 mM phosphate buffer (pH6.0) containing 0.1% NP40. The flow through fraction contained pure HN protein.

As shown in Fig.1, the purified preparation of F protein give two main bands (F_1 and F_2) and HN a single sharp band under reducing conditions on sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis.

Isolation of fragment A of diphtheria toxin and assay of its NAD:EF2-ADPR transferase activity

CRM 22, a mutant protein produced by the C7 (β22) strain, that is immunologically identical to fragment A of diphtheria toxin, was purified by DE-52 and Sephadex G-150 chromatography as described previously (51). The NAD:EF2-ADPR transferase activity of each sample was assayed by the method of Gill and Pappenheimer (11), except that EF2 was partially purified on DEAE cellulose (51).

Polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (56) in 5 min diameter rod of 7.5% acrylamide gel. Gels were stained for protein with Coomassie brilliant blue.

Determination of protein

Protein was determined by the method of Lowry, et al. (24).

Electron microscopic obserbation

The procedure of negative staining was described previously (51). Samples were obserbed under a Hitachi 12A type electron microscope at 75 kV.

Antibody against HN or F protein

Samples of 1 mg of HN or F protein, purified as described above, were mixed with complete Freund's adjuvant and injected into rabbits subcutaneously. Three weeks later a booster injection of 0.5 mg protein in complete Freund's adjuvant was given. Booster injections were repeated at intervals until a suitable antibody titer

was obtained. Antiserum was incubated at 56°C for 30 min to inactivate complement and the globulin fraction was prepared by precipitation with 0.33 saturated ammonium sulfate and dialysis of the resulting precipitate against PBS (33).

Monoclonal antibodies HN-1 and HN-2 were obtained from ascitic fluid of pristan-treated mice injected with about 2 x 106 cells of the appropriate hybridoma cell line. The antibodies were purified by affinity chromatography using HN protein conjugated to Sepharose 4B (30).

Preparation of fragment A-containing liposomes

A mixture of 400 mg of egg yolk lecithin and 50 mg of cholesterol in chloroform was lyophilized in the presence of nitrogen gas. Then 2 mg of lyophilized lecithin-cholesterol, 2 mg of fragment A and purified F and HN, as described in the figure captions, were mixed in a final volume of 1 ml and 25 µl of 20% NP40 was added to a mixture to solubilize them. The solution was dialyzed at 4°C against 10 mM phosphate buffer (pH7.2) containing 0.3 M sucrose and 1 mM KCl for 7 days. The resulting lipid vesicles were purified by chromatography of the dialyzed turbid solution on a column of Bio-Gel A50m. The void volume was collected and purified further by centrifugation through a linear sucrose gradient (12-40% wt/vol) in 10 mM phosphate buffer (pH 7.2) over a cushion of 60% sucrose in the same buffer, as described previously (51).

Preparation of liposomes associated with wheat germ agglutinin (WGA).

N-[3-(2-pyridyldithio)propionyl]phosphatidylethanolamine (PDP-PE) was synthesized from (N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) and phosphatidylethanolamine and purified by the method of Martin, et al. (26). 1 ml of Wheat germ agglutinin (WGA) (1 mg/ml) in 0.15 M NaCl, 10 mM phosphate buffer (pH7.4), 1 mM EDTA was mixed with 5 µl of 50 mM SPDP in dioxane, and incubated for 1 hr at room temperature. The mixture was then purified by chromatography

on a Sephadex G25 column. Protein fraction was stocked (WGA-PDP).

4 mg of lyophilized lecithin-cholesterol (8:1 w/w), 100 μg of PDP-PE and 0.6 mg of fragment A of diphtheria toxin was suspended in 1 ml of 10 mM phosphate buffer containing 0.3 M sucrose, 1 mM KCl and 5 mM dithiothreitol (DTT), and then 30 μl of 20 % NP40 was added to the mixture. For making liposomes containing CRM45, 0.6 mg of CRM45 and 1 mM bicarbonate buffer (pH8.5 at 0 °C) was used instead of fragment A and phosphate buffer, respectively. This solution was mixed with Bio-Beads SM-2 and was shaken at 4 °C for over night. The resulting lipid vesicles were mixed with 10 μl of 0.5 M DTT, incubated for 1 hr at room temperature and applied to a Bio-Gel A150m column. The void volume was collected. 250 μg of WGA-PDP was added immediately to this liposome solution, which was then incubated for 1 hr at room temperature. Liposomes were further purified by a Bio-Gel A150m culumn.

Preparation of hybrid toxin

WGA-PDP was prepared as described above. 1 mg of fragment A was reduced with 10 mM DTT at pH8.0 for 1 hr at room temperature, then applied to a Sephadex G25 column equilibrated with 10 mM phosphate buffer (pH7.4), 0.15 M NaCl and 1 mM EDTA. The protein fraction (the void volume) was collected, and 250 μg of WGA-PDP was added immediately. This mixture was incubated for 1 hr at room temperature, then applied to a Sephadex G150 column and fractions containing hybrid toxin were collected.

Binding assay of liposomes to cells

200 μl of liposomes containing ^{125}I-fragment A suspended in PBS was mixed with 200 μl of packed Ehrlich ascites tumor (EAT) cells and vortexed. This mixture was incubated at 4 °C for 30 min, then at 37 °C for 30 min. Cells were washed four times with cold PBS containing 2 mg of bovine serum albumin (BSA) per ml by centrifugation. Then cells were counted in a γ counter.

Assay of the rate of protein synthesis

Cells were plated in 24 well tissue culture plates and incubated for 8 hr in Eagle's MEM containing 10% calf serum. The medium was replaced with liposomes suspended in MEM and HVJ suspended in MEM (2,000 HAU/ml), and the cells were then incubated at 4 °C as described in figure legends. After incubation at 4 °C, the medium was replaced with 0.3 ml of MEM and cells were incubated at 37 °C for 30 min. Then 0.3 ml of MEM containing 10% calf serum was added to each plate and incubation was continued at 37 °C for 14 hr. Cells were then labeled with 2 μCi/ml of [^{3}H]leucine for 60 min. The medium was removed and 0.1 N NaOH was added. The cells were collected and treated with 10% trichloroacetic acid, and the precipitate was collected on a glass filter, dried and counted in a liquid scintillation system. The rate of protein synthesis was expressed as a percentage of the value obtained in the control cultures without liposomes (29). Control values were 10,000 to 40,000 cpm.

Assay of cytotoxicity of lipid vesicles containing fragment A

Suspensions of 300 L cells in 2 ml of Eagle's minimum essential medium (MEM) containing 10% calf serum were incubated in 30-mm plastic Petri dishes for 7.5 hr at 37 °C. The cells were washed once with 2 ml of chilled balanced salt solution (BSS) (140 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, buffered with 10 mM Tris-HCl at pH 7.6 containing 2 mM $CaCl_2$), and then 0.5 ml of a chilled suspension of lipid vesicles containing fragment A was added. The dishes were kept for 20 min at 4 °C, warmed to 37 °C, and incubated for a further 20 min. Then, 2 ml of culture medium was added and incubation was continued for 1 hr at 37 °C. The medium was replaced by fresh medium and the cells were cultured for 7 days. The cells were finally fixed with methanol and stained with Giemsa. Experiments were performed using duplicate dishes.

Assay of cytotoxicity of Fragment A-containing liposomes for SSPE cells

SSPE cells were plated on HEL cells in 35 mm dishes. Three days after infection, when syncytial giant cells had formed, the cultures were washed once with 2 ml of Hank's salt solution (HBSS) and treated with 0.2 ml portions of serial 4-fold dilutions of various liposome preparations at 37 °C in a CO_2 incubator. The cells were exposed to liposomes for 2 hr unless stated otherwise. Liposomes were sterilized by ultraviolet radiation and diluted in 199/MEM just before use. The cultures, with or without washing to remove the liposomes, were then fed with 2.5 ml of medium 199/MEM supplemented with 5% fetal bovine serum and incubated two days at 37 °C. Infectivity of the liposome treated cultures was then measured by an infectious center assay (54). Each culture was dispersed with 0.25% trypsin in a 1:1,500 versene solution. The cells were suspended in one ml of medium. Cells from duplicate cultures were pooled and 0.2 ml was added to duplicate CV-1 cell monolayers grown in 60 mm plastic dishes containing 6 ml fresh medium 199/MEM with 3% fetal bovine serum. After 3 days at 37 °C the CV-1 cells were stained with Giemsa solution and plaque-forming units (PFU) per dish. Uninfected HEL cell cultures were similarly treated with liposomes and cultured. After dispersion the viable cells were counted after staining with 0.5% trypan blue.

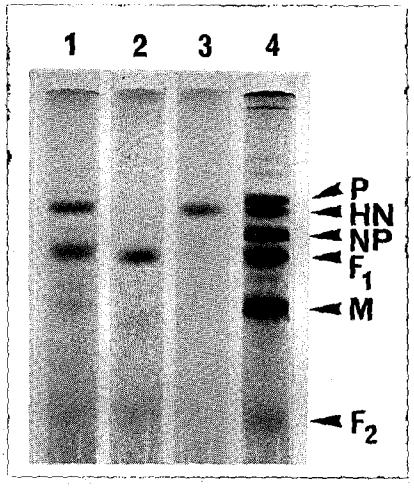

FIGURE 1

Analysis of purified HVJ envelope glycoproteins by SDS polyacrylamide gel electrophoresis. Lane 1, solubilized HVJ envelope glycoproteins; lane 2, purified F protein; lane 3, purified HN protein; lane 4, whole HVJ proteins. Each protein sample was reduced with dithiothreitol (DTT), denaturated with 1% SDS, and applied to 7.5% polyacrylamide gel.

PART 1

SUMMARY

The biological activity of two glycoproteins, hemagglutinin and neuraminidase (HN) and fusion (F) proteins, of Sendai virus (HVJ) were studied using purified proteins. The proteins were purified by chromatography on DEAE and CM cellulose in the presence of Nonidet P-40 (NP40). The glycoproteins were reconstituted at various ratios of F to HN into lipid vesicles containing fragment A of diphtheria toxin. The associasion of HN and F proteins with the vesicles was confirmed by electromicroscopy and sucrose density gradient centrifugation. The cytotoxic activity of vesicles containing fragment A on fusion with L cells was determined by measuring colony formation of the cells. It was found that for maximum cytotoxic activity of the vesicles, there was an optimal ratio of F to HN of two. This suggests that HN is not merely the initial binding site to the cell surface, and that interactions between HN and F proteins on the virus surface may be important for the biological activities of these proteins on the cells.

INTRODUCTION

Since intact Sendai virus (HVJ) virions release their nucleoproteins into the cytoplasm after fusion with mammalian cells, it has been shown by measuring inhibition of colony formation that non-toxic fragment A of diphtheria toxin within lipid vesicles associated with HVJ glycoproteins can be introduced efficiently into the cytoplasm of L cells (51). As described in GENERAL INTRODUCTION, the toxicity to mammalian cells of lipid vesicles containing fragment A (i.e., activity for introduction of molecules from the lipid vesicles into the cell cytoplasm as the result of fusion between the cell membrane and vesicle membrane) indicates the efficiency of cell membrane-vesicle membrane fusion.

The glycoproteins of HVJ are composed of F and HN proteins. F is important in fusion of viral envelope to the cell membrane, virus-induced cell fusion and hemolysis (14, 15, 42). HN is considered to be the initial binding site of the virion to the cell surface (42, 48). For further studies on the biological activity of these viral envelope glycoproteins, HVJ envelope glycoproteins were separated by affinity chromatography on Fetuin-Sepharose 4B (41, 42). Hsu, et al. (19) reported reconstitution of lipid vesicles containing HVJ envelope proteins (F and HN) that had been purified separately on Fetuin-Sepharose. They described a method for reconstitution of lipid vesicles in cholate solution and the characters of these lipid vesicles investigated by electronmicroscopy and equilibrium sedimentation. They found that lipid vesicles with F protein did not have a hemolysing activity, and that this activity was restored by addition of wheat germ agglutinin (WGA). However, they discussed this phenomenon only from the view point of lack of binding activity of the vesicles to the cell surface.

We developed a simple method for protein purification; namely, by column chromatographies on DEAE cellulose and CM cellulose. Though hemolytic activity

has been determined as one of the activities of the glycoproteins (19, 42), we used in this paper the resulting purified glycoproeins at various ratios to reconstitute lipid vesicles containing fragment A and determined the cytotoxicities of the vesicles on fusion with L cells by measuring the inhibition of colony formation. The results showed that the ratio of the two glycoproteins was important for efficient cell membrane-vesicle membrane fusion.

RESULTS

Characterization of lipid vesicles reconstituted from F, HN and lipids

For investigation of the biological function of viral envelope proteins, lipid vesicles were reconstituted with HN or F proteins. The reconstituted vesicles were first examined by electron microscopy. Either F or HN protein could form vesicles with lipid, the sizes of which were nearly equal to that of large HVJ virion. These vesicles had a lot of spike-like structures on both sides of the membrane, as obserbed on reconstitution with HVJ envelopes (51) (Fig.2).

To confirm the association of F and HN with lipid vesicles and the enclosure of fragment A in these vesicles, we add antibodies against each glycoprotein to purified lipid vesicles associated with glycoproteins and containing fragment A, and examined their sedimentation profiles in sucrose gradient before and after addition of antibody. After addition of anti-F antibody to lipid vesicles associated with HVJ envelopes or with F, and after addition of anti-HN antibody to lipid vesicles with HN, most activity of fragment A was recovered in the sedimented fraction, whereas before addition of antibody, it was obserbed in the top fraction (Fig.3). So we concluded that both F and HN protein can associate with lipid vesicles and that fragment A was mainly present in the lipid vesicles not outside of them.

We then reconstitute the vesicles with various ratios of HN protein to F protein, and examine the ratios of proteins associated with purified vesicles. The vesicles purified as described in MATERIALS AND METHODS were solubilized in buffer containing 1% SDS, then analyzed by SDS polyacrylamide gel electrophoresis. The ratios of proteins associated with vesicles were almost the same as the ratios before reconstitution (Fig.4). Furthermore, in these vesicles the hemagglutinating activity and neuraminidase activity associated with the surface of lipid vesicles increased as the amount of HN protein in the reconstitution

mixture was increased (Fig.5). In the presence of a constant amount of HN protein, the hemagglutinating activity and neuraminidase activity did not change when the amount of F protein was varied (data not shown). In all cases, the total recovery of neuraminidase activity on surface of lipid vesicles was about 12-32%. Thus it is likely that the F protein does neither participate in the hemagglutinating activity or in the neuraminidase activity.

Biological activity of lipid vesicles reconstituted with F, HN and lipids.

To determine the fusion of reconstituted vesicles with the cell membrane, we measured the toxicity of lipid vesicles containing fragment A of diphtheria toxin. Fragment A has no toxicity when it is present outside of the cell membrane (50), but as little as one molecule can kill a mammalian cell when it is introduce the cytoplasm (58). So, fusion of lipid vesicles with the cell membrane can be measured by determining the toxicity of vesicles containing fragment A; i.e., by measuring the ability of lipid vesicles to introduce their contents into cells.

We examined the toxicity of lipid vesicles containing fragment A reconstituted from various ratios of HN and F. As shown in Fig.6, lipid vesicles reconstituted without F or HN were not toxic. The toxicity was maximal when the ratio of F to HN was about 2; with other ratios of the two proteins, the toxicity was less than 50% of the maximum. Empty lipid vesicles with HVJ envelope glycoproteins had no toxicity (data not shown). Similar results were obtained using lipid prepared from the membranes of Ehrlich tumor cells in place of lecithin and cholesterol for reconstitution (data not shown). These vesicles, however, showed lower hemolytic activity than native virions. At all ratios of F and HN, the hemolytic activity was less than 5% of that of native virions.

These facts suggest that F and HN are both necessary for vesicle membrane-cell membrane fusion (introduction of materials in vesicles into the cells), that

there is an optimal ratio of F to HN, and that some optimal conformation of the two envelope glycoproteins is probably important for efficient fusion.

DISCUSSION

The method for preparation of HVJ envelope glycoproteins using ion-exchange resins described in this paper is very much simple. We analyzed the carbohydrate structures of F and HN protein prepared by this method, finding much difference between them (60).

Various methods have been used to measure the efficiency of fusion of cell membrane with lipid vesicle membranes (e.g., measurement of hemolytic activity and the spin label method) but the method of measuring the efficiency of introduction of fragment A of diphtheria toxin enclosed in lipid vesicles into the cytoplasm is the most sensitive and logical one method. Our method is based on the fact that fragment A is not toxic to sensitive cells when present in the culture medium (50), but kills mammalian cells when as little as one molecule is introduced into the cytoplasm (58). The sensitivity and utility of this method is indicated by the fact that various kinds of lipid vesicles showing little difference in hemolysing activity show great differences when measured by this method.

We first thought that F (cleaved form) causes leak of fragment A from vesicles through the membrane of a lipid bilayer, whereas F_0 does not. But F (cleaved form) alone, which was well associated with lipid vesicles, did not cause leak of fragment A from vesicles incubated at 4-0 °C for several days under our conditions.

We found that there is an optimum ratio of F protein to HN protein (about 2) for efficient fusion of vesicles with the cell membrane. This ratio is consistent with the ratio of F to HN in intact virions (about 1.7) caluculated from the ratio of intensities of staining with Coomassie brilliant blue of the two proteins on SDS polyacrylamide gel electrophoresis (our unpublished results). Furthermore, even if the lipid vesicles are associated with sufficient HN protein, there is an optimal

ratio of F to HN for efficient fusion (Fig.6B). From our data and the results reported by Miura et al. (30), that a monoclonal anti-HN antibody exists that does not inhibit the hemagglutinating activity of HVJ but inhibits cell fusion, we could speculate that HN protein probably had a second binding site for expression of the function of F protein after initial binding to the cell surface using neuraminidase activity, and that F protein might need to interact with HN protein to induce fusion.

We know little, however, about the conformational relation between F and HN in native virions. This problem needs further biochemical and biological investigation.

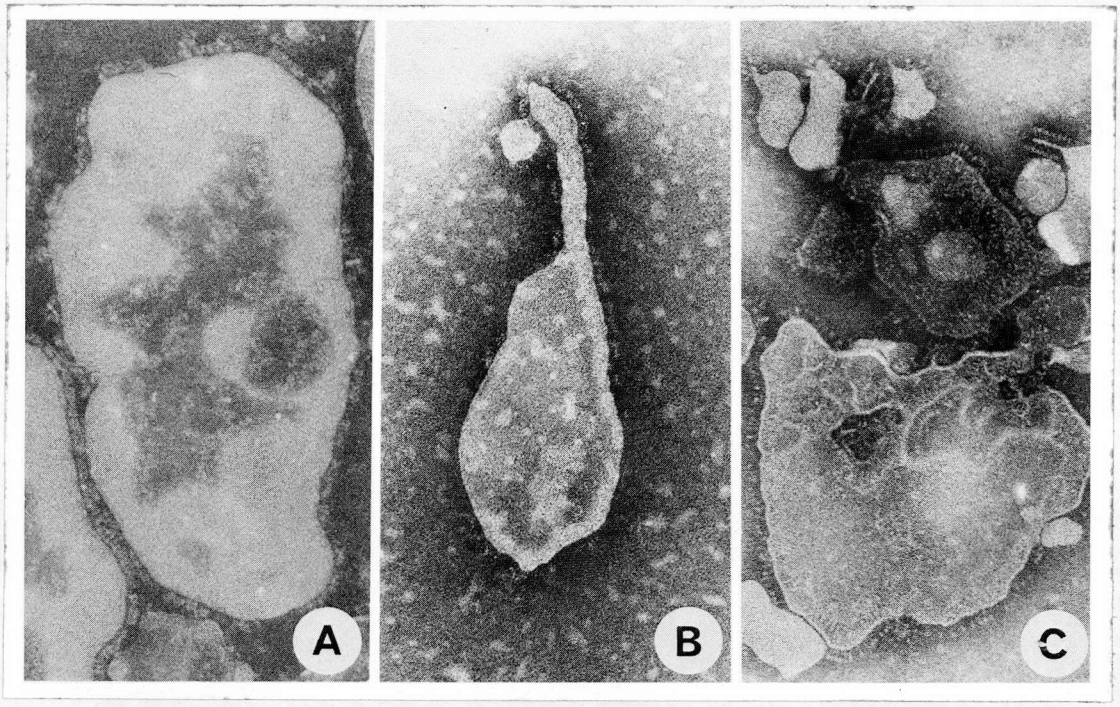

FIGURE 2

Electron microscopic appearance of liposome reconstituted from purified HVJ envelope glycoproteins. (A) liposome with HVJ envelope (F and HN), (B) liposome with HN, (C) liposome with F. x 100,000.

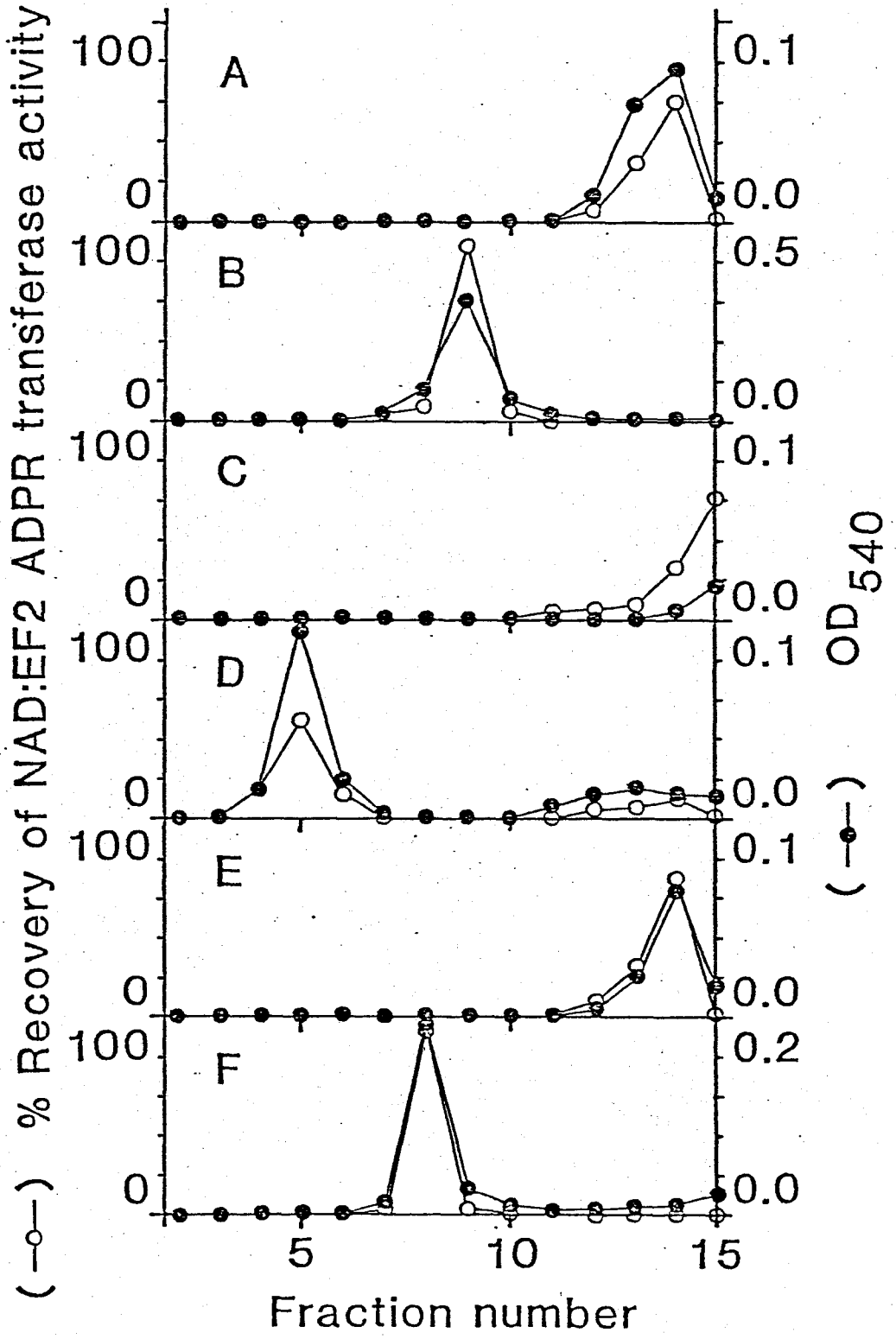
A
B
C
D
E
F
100
0
0.1
0.0
0.5
0.2
5
10
15
Fraction number
(—o—) % Recovery of NAD:EF2 ADPR transferase activity
(—●—) OD540

FIGURE 3

Sedimentation analysis of antibody-treated liposomes containing fragment A. (A)(B) liposomes with F, (C)(D) liposomes with HN or (E)(F) liposomes with HVJ envelopes, were treated with anti-F antibody (B)(F), anti-HN antibody (D), or control immunogloblin (A)(C)(E) and then subjected to linear sucrose gradient (12-40 %) cetrifugation. Fractions of 1 ml were collected from the bottom. The absorbance at 540 nm was measured, then NAD:EF2 ADPR transferase activity was measured after treatment with 0.5% NP40.

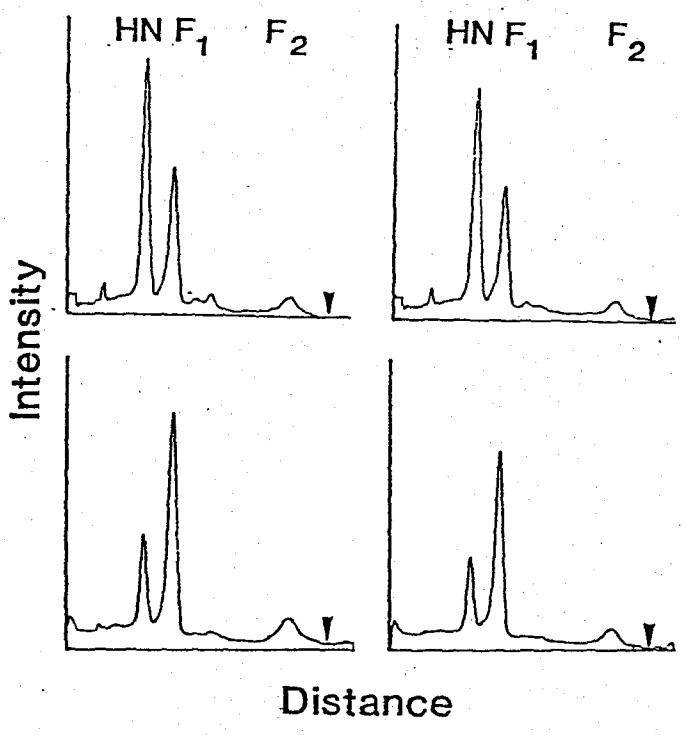
HN F1 F2
HN F1 F2
Intensity
Distance

FIGURE 4

SDS-polyacrylamide gel electrophoretic analysis of purified vesicles associated with F and HN protein. Lipid vesicles were reconstituted at the ratio of F to HN of 0.5 (A)(B) or 2 (C)(D). after purification, vesicles were solubilyzed in buffer containing 1% SDS, then analyzed by SDS polyacrylamide gel electrophoresis (B)(D). Protein mixture before reconstitution were also analyzed (A)(C). Gels were stained with Coomassie blilliant blue and scanned at 550 nm. The arrows denote the position of Bromphenol blue.

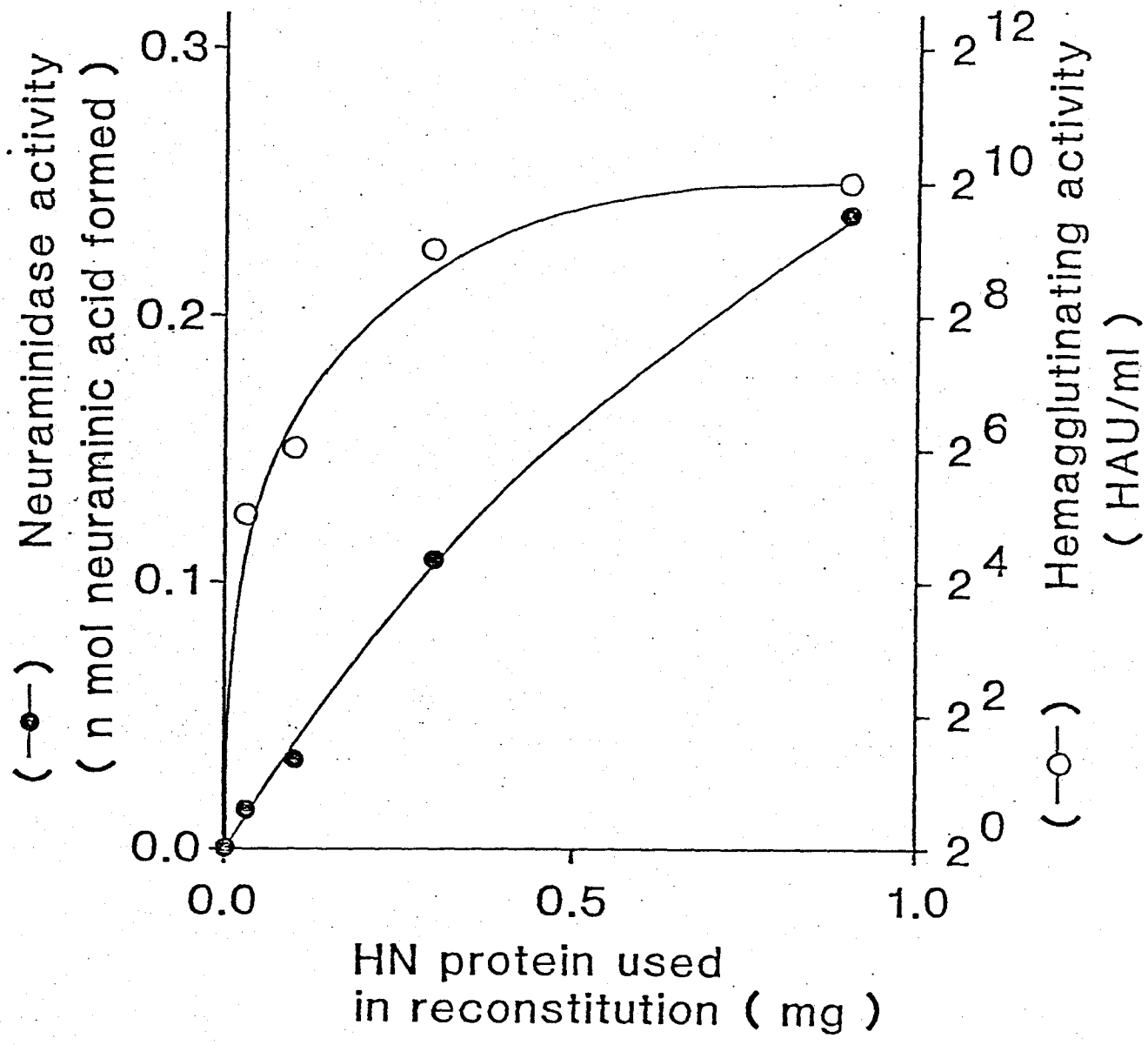
(—●—) Neuraminidase activity
(n mol neuraminic acid formed)
0.3
0.2
0.1
0.0
(—○—) Hemagglutinating activity
(HAU/ml)
2^{12}
2^{10}
2^{8}
2^{6}
2^{4}
2^{2}
2^{0}
0.0
0.5
1.0
HN protein used
in reconstitution (mg)

FIGURE 5

Neuraminidase and hemagglutinating activity associated with the surface of lipid vesicles reconstituted with HVJ envelope proteins. Volumes of 10 ul and 500 ul of liposome solution (OD_{540}=0.01) were used for assay of neuraminidase activity and hemagglutinating activity, respectively. Liposomes were reconstituted with the indicated amount of HN, 0.6 mg of F and 2 mg of lipids as described in MATERIALS AND METHODS.

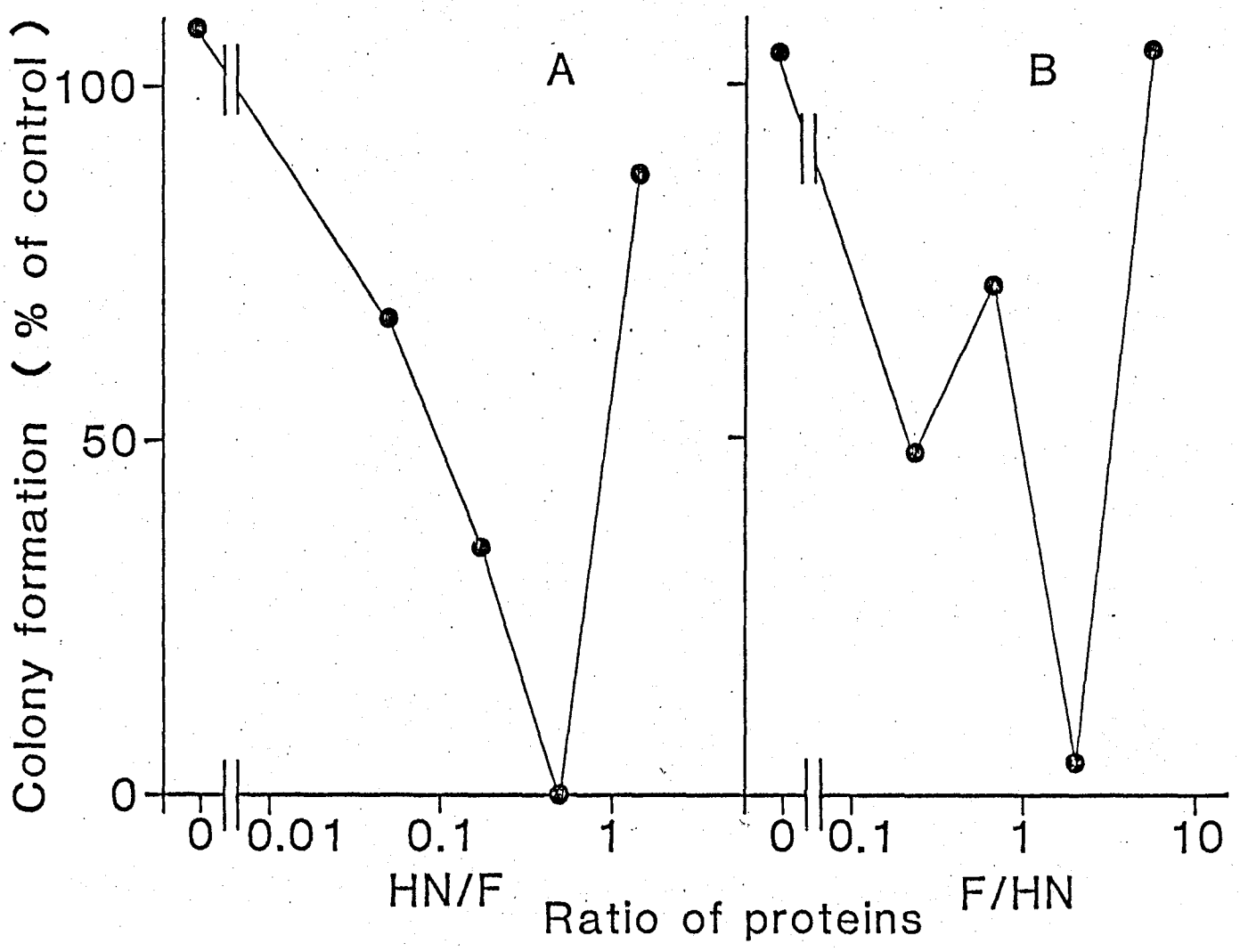
Colony formation (% of control)
100
50
0
A
B
0
0.01
0.1
1
0
0.1
1
10
HN/F
F/HN
Ratio of proteins

FIGURE 6

Toxicity of liposomes containing fragment A to L cells. Liposomes were reconstituted with 0.6 mg of F protein, 0-0.9 mg of HN protein and 2 mg of lipids (A), or with 0.3 mg of HN protein, 0-1.8 mg of F protein and 2 mg of lipids (B). They were purified and their toxicity on L cells was measured as described in MATERIALS AND METHODS. The liposomes were adjusted an absorbance of 0.13 at 540 nm. Each sample contained about 2 ug/ml of fragment A. Control dishes contained 119 (A) and 140 (B) colonies.

PART 2

SUMMARY

The changes in cytoplasmic membranes of cells treated with HVJ were studied. Naked liposomes containing fragment A and liposomes containing fragment A and associated with wheat germ agglutinin (WGA) were prepared. These liposomes were not toxic for untreated L cells, but were toxic when cells were treated by HVJ. Liposomes containing CRM45, a kind of mutant protein of diphtheria toxin, instead of fragment A, also were toxic in the presence of ammonium chloride, an inhibitor of penetration of CRM45 through the lysosomal pathway, only when cells were treated by HVJ. The toxicity of hybrid toxin consisting of WGA and fragment A of diphtheria toxin for L cells was not increased by addition of HVJ. The possibility that the cell membrane was changed by HVJ to allow fusion with liposomes is discussed.

INTRODUCTION

Though cell fusion occurs subsequently to viral envelope fusion, it was shown that envelope fusion (fusion between viral envelope and cell membrane) was separable from cell-cell fusion (fusion between cell membranes) by Miyake et al. (31). Thus, some changes in the cell membrane after envelope fusion were thought to be important for inducing cell-cell fusion. The fact that cytocharasin D inhibits cell-cell fusion but does not inhibit envelope fusion suggests that changes in the cell membrane caused by the microfilament system would have an important role in cell-cell fusion.

Several changes in cell membrane were observed when cells were treated with HVJ. One of them, which seems most important, is a clustering of intramembrane particles (IMP) observed in human erythrocytes (2, 44) and Ehrlich ascites tumor (EAT) cells (21) at cell fusion. This phenomenon is known to be temperature dependent and inhibited by cytochalasin D and glucose at high concentration (21), which inhibit cell-cell fusion but do not inhibit envelope fusion in EAT cells, so it is strongly suggested that this structural change in the cell membrane is an essential step in cell-cell fusion. Judging from observations in the electronmicroscope, fusion between cell membranes occurs in areas where the membranes of neighboring cells are in contact near the places of virus adsorption not precisely at the position of HVJ on the membrane (21). The cluster-free area observed at cell fusion might be that close contact area.

Another observation of change in the cell membrane during the cell fusion process was studied using a phosphatidylcholine spin label. Maeda, et al. (27) reported that when spin labeled influenza virus were absorbed to the erythrocyte surface, the disappearance of the exchange broadening in the electron spin resonance (ESR) spectrum of virus (i.e., transfer and intermixing of phospholipid

molecules between viral envelope and erythrocyte membrane) was observed only slightly, whereas it was greatly enhanced by treatment with HVJ. This phenomenon indicates that some changes were caused by HVJ in erythrocyte membrane which allow the cell membrane to fuse with influenza virus.

In living cell systems, we had no direct evidence whether the cell membrane has been changed by HVJ treatment to such that another membrane can fuse with it. I examine this problem using liposomes containing fragment A of diphtheria toxin. As described in GENERAL INTRODUCTION, if these vesicles fuse to the cell membrane, fragment A can be delivered into cytoplasm to inhibit protein synthesis of the cell (the cell is killed as a result). In this paper, I demonstrate that these liposomes can fuse with cell surface only when cells are treated by HVJ.

RESULTS

Interaction of naked liposomes with HVJ treated cells

Under normal conditions, mammalian cells do not fuse with other cells or with liposomes, unless the liposome itself has fusion activity, as shown in chapter 1. Considering the evidence described in the introduction, the cell surface of HVJ-treated cells seems to have changed to be ready to fuse with other cell membranes or lipid vesicles, so we first examined whether naked liposomes can fuse to cells treated with HVJ. Mouse L cells were allowed to absorb HVJ at 4°C for 20 min, and then they were washed once with PBS. Liposomes containing fragment A of diphtheria toxin suspended in PBS were added to cell culture and it was incubated for 15 min at 37°C. Then liposomes were washed out and replaced with fresh medium. In another case, cells were preincubated with medium for 15 min at 37°C before addition of liposomes. Table 1 shows that inhibition of colony formation of cells occured when cells were treated with HVJ and liposome containing fragment A of diphtheria toxin. In the absence of HVJ, liposomes did not affect cell viability. The inhibition of colony formation was less when the liposome suspension was added after pre-incubation of HVJ-absorbed cells in medium for 15 min at 37°C. HVJ alone did not decrease the number of colonies formed by L cells. These data show that after cells were treated with HVJ there is a short period when cells are able to fuse with naked liposomes. In this experiment, inhibition of colony formation was also observed at a lower degree when HVJ-absorbed cells were treated with the solution containing empty liposomes and fragment A outside of vesicles. This is because macromolecules like fragment A and T4 endonuclease V can penetrate the cell membrane when cells are incubated with HVJ, as described previously (59, 47). In the system using HVJ-treated cells and naked liposomes, however, a small amount of free fragment A outside of vesicles was present, so to

obtain clearer results I used a system consisting of HVJ-treated cells and liposomes with wheat germ agglutinin on their surface, which have the ability to bind to the cell surface.

Interaction of wheat germ agglutinin (WGA)-conjugated liposomes with HVJ-treated cells.

Methods for conjugating proteins to liposome surfaces covalently by a disulfide bond were first reported by Martin, et al. (26). When liposomes were conjugated with lectins or antibodies against the cell surface, they can bind to cells which have receptors for lectins or antigen on their surface. Table 2 shows the binding to Ehrlich ascites tumor (EAT) cells of liposomes which contained ^{125}I-labeled fragment A inside of vesicles that were conjugated with WGA. When these liposomes were incubated with EAT cells, more than 87 % of the fragment A was associated with cells, and this association was inhibited by reducing agent which cleaves the disulfide bond between WGA and liposome. Fragment A itself does not associate with cells. These data showed that fragment A was trapped within vesicles and that these vesicles could associate with the cell surface by the binding activity of WGA. Next, I examined the fusion activity of HVJ-treated cells with the liposomes containing fragment A and associated with WGA. Cells were incubated first with the liposomes and then HVJ at 4°C for 30 min each, and the medium was replaced with fresh medium after each incubation. The cells were incubated for 14 hours at 37°C and then the rate of protein synthesis was measured. Under these conditions, fragment A outside of vesicles was washed out. Table 3 shows that protein synthesis was inhibited when cells were treated with liposomes and HVJ, but was not inhibited when cells were treated only with liposomes. No inhibition of protein synthesis was observed when cells were treated with HVJ and empty liposomes associated with WGA (no fragment A). These data

indicate that fragment A contained inside the lipid vesicles penetrated into cell cytoplasm as a result of the treatment of HVJ.

Two possibilities might be considered to explain this phenomenon. One is that liposomes fused with the cell membrane because the state of cell membrane was changed by HVJ to allow fusion with liposomes. Another is that liposomes were taken up by endocytosis and then fragment A in the endocytotic vesicles penetrated the membrane to reach the cytosol after fusion with a lysosome, because activation of endocytosis might occur as a result of the dramatic change in the cell cytoskelton caused by HVJ (20). In order to distinguish between these possibilities, I used liposomes containing CRM45, a mutant protein of diphtheria toxin, instead of fragment A. CRM45 is a non-toxic mutant protein with a molecular weight of about 45,000, which lacks 17,000 C terminal region of the toxin, and does not bind to receptors (50). This protein inhibits protein synthesis when introduced into cytoplasm, and also is toxic for both diphtheria toxin-sensitive and insensitive cultured cells when it is present at high concentration in the culture medium (50). The toxicity of CRM45 in culture medium was inhibited by ammonium chloride (Fig.7). Ammonium chloride was reported to inhibit the action of diphtheria toxin (29, 39, 6), so this reagent was thought to inhibit the passing of CRM45 through lysosomal membrane at low pH. I examined the inhibition of protein synthesis by liposomes containing CRM45 and associated with WGA in the presence of HVJ and ammonium chloride. Table 4 shows the protein synthesis of cells treated with liposomes containing CRM45 and HVJ in the presence or absence of ammonium chloride. When cells were treated with liposomes containing CRM45 and HVJ, protein synthesis decreased regardlessly of the presence of ammonium chloride. This suggests that penetration of CRM45 from the liposome to the cytoplasm is not through the lysosomal pathway.

In addition, we examined whether HVJ has an effect on the toxicity for L

cells of a hybrid toxin consisting of WGA and fragment A of diphtheria toxin. This hybrid toxin was considered to inhibit protein synthesis by penetration into the cytoplasm through the lysosomal pathway when it is present in the medium at high concentration. No difference was observed in the toxicity of hybrid toxin for L cells whether cells were treated by HVJ or not (Fig.8). HVJ thus seems to have no effect on the uptake of molecules by endocytosis.

DISCUSSION

In this report, I have shown that the liposomes containing fragment A were toxic for cells when the cells were treated with HVJ. This phenomenon is due to the introduction of fragment A inside vesicles into the cytoplasm by direct fusion of liposomes and the cell membrane, because the penetration of fragment A through lysosomal pathway was ruled out by the experiments using the liposomes containing CRM45 and using hybrid toxin. As liposomes containing fragment A and associated with WGA cannot fuse with cell membrane under ordinary conditions, the fusion of liposomes to cell membrane was without doubt due to changes in cell membrane caused by HVJ. This report is the first to report an artificial changes in cell membranes caused by HVJ that allow fusion with another lipid membrane in a living cell system.

This change in cell membrane corresponds to the formation of clusters of intramembrane particles (IMP) during cell fusion observed by electromicroscopy, as both were caused by HVJ and were inhibited by cytochalasin D (21) (data not shown). Sekiguchi et al. (44), showed that the clustering of IMP was indispensable step in cell fusion in the erythrocyte system. They reported that when anti-spectrin antibody was introduced into erythrocyte ghosts, these ghosts cannot fuse with other cells. At the same time, it was observed by electromicroscopy that the clustering of IMP, which was observed in ordinary cell fusion, was inhibited. When antibody was used in F_{ab} form, inhibition of cell fusion and clustering of IMP was not observed. They suggested that this inhibition of cell fusion and clustering of IMP was due to the cross linkage of spectrin by antibody, and that spectrin movement caused by HVJ played an important role in fusion of erythrocyte ghosts. In the living cell system, the changes in the cell membrane caused by cytoskeltal proteins will also be important in cell fusion. The exposure oflipid bylayer

caused by cluster formation might be important for fusion with othermembranes.

This phenomenon was also useful as the fourth method for the introduction of macromolecules into living cells. Three methods have already been described for introduction of macromolecules into living cells: direct microinjection with micropipettes (5), red-cell mediated microinjection (9, 25, 43), and the method using liposomes associated with HVJ envelope glycoproteins (51). The fourth method using liposomes and HVJ has the advantage that we can easily load molecules such as DNA into liposomes under sterile condition, and that macromolecules might be able to be introduced into specific cells which have some special antigen using the liposomes conjugated with the antibody against this antigen. Furthermore, when receptors for some molecules associated with liposomes might be able to be transplanted to cell membrane. Developement of this fourth method for introduction of macromolecules into cells is now in progress.

	COLONY NO. PER DISH (% OF CONTROL)	
	0 min	15 min
Liposome cont. F.A.	19	62
Empty Liposome +F.A.(0.9μg/ml)	68	102
PBS	100	100

TABLE 1

Toxicity of naked liposomes containing fragment A for L cells treated with HVJ. L cells were treated with HVJ (2,000 HAU) in one ml of BSS for 20 min at 4 °C, and then washed out. After treatment with liposomes, cells were cultured as described in MATERIALS AND METHODS. (0 min) Cells were incubated with liposomes for 15 min at 37 °C just after HVJ treatment; (15 min) cells were incubated in medium for 15 min at 37 °C after HVJ treatment, then incubated with liposomes for 15 min at 37 °C. (A) liposomes containing 2.7 μg fragment A per ml inside and 0.9 μg fragment A per ml outside of vesicles; (B) Empty liposomes and free fragment A (0.9 μg/ml) outside of vesicles; (C) PBS. Control dishes contained (0 min) 93 (15 min) 121 colonies.

		Liposome binding (% of control)
Liposome with WGA	-	87.9
	DTT	3.6
Liposome without WGA	-	7.2

TABLE 2

Binding activity of naked liposomes and liposomes associated with WGA to Ehrlich ascites tumor (EAT) cells. Liposomes associated with WGA and containing ^{125}I fragment A (OD_{540} = 0.160, 16,000 cpm/ml) and naked liposomes containing ^{125}I fragment A (OD_{540} = 0.160, 16,000 cpm/ml) were used for binding assay in the presence or absence of 20 mM DTT as described in MATERIALS AND METHODS.

PROTEIN SYNTHESIS (% OF CONTROL)

	ng Fragment A / ml			
	460	46	4.6	0
HVJ(+)	16.0	71.1	87.8	100.0
HVJ(-)	82.1	89.6	88.6	100.0

TABLE 3

Toxicity of fragment A-containing liposomes associated with WGA for L cells treated with HVJ. L cells were incubated with each concentration of fragment A-containing liposomes associated with WGA (2.3 μg fragment A per ml at OD_{540} = 0.50) for 30 min at 0 °C, then washed. Cells were further incubated for 30 min at 4 °C with HVJ (2,000 HAU per one ml of MEM) or MEM. Then the medium was replaced with MEM. After the incubation for 30 min at 37 °C, MEM containing 10% calf serum was added. The rate of protein synthesis was determined as described in MATERIALS AND METHODS.

		PROTEIN SYNTHESIS (% OF CONTROL)		
		Liposome containing——		
NH_4Cl	HVJ	CRM45	Fragment A	Nothing
+	+	5.8	5.3	93.4
+	-	101.6	113.9	103.0
-	+	33.3	27.6	95.9
-	-	93.1	99.3	103.5

TABLE 4

Effect of ammonium chloride on the toxicity of liposomes for HVJ treated cells. Liposomes containing CRM45 and associated with WGA (500 ng CRM45 per ml, OD_{540} = 0.12) and liposomes containing fragment A and associated with WGA (460 ng fragment A per ml, OD_{540} = 0.10) were incubated with cells for 30 min at 4°C. The cells were washed, and were incubated with HVJ (2,000 HAU per ml of MEM) for 30 min at 4°C. The medium was replaced with MEM, and incubation was continued for 30 min at 37°C. Then MEM containing 10% calf serum was added to each culture and incubation was continued for 14 hr at 37°C. The rate of protein synthesis was determined as described in MATERIALS AND METHODS. Cells were incubated with 5 mM ammonium chloride (NH_4Cl +) or without ammonium chloride (NH_4Cl -) through out the process.

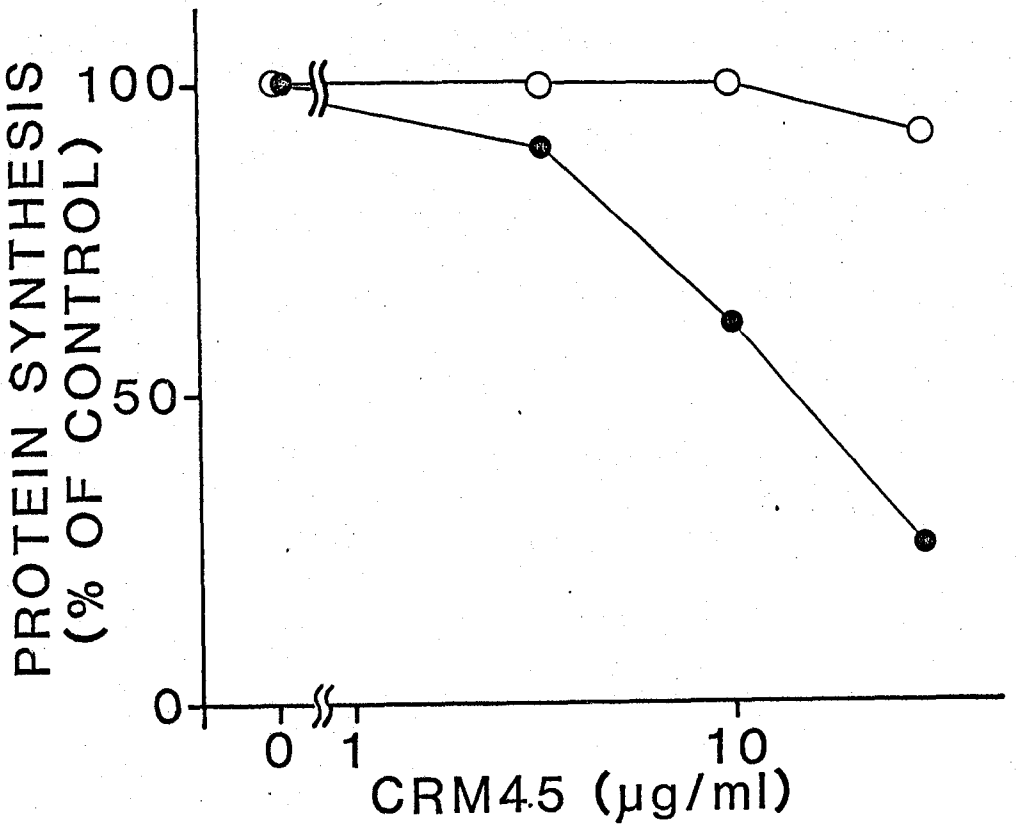
PROTEIN SYNTHESIS
(% OF CONTROL)
100
50
0
0
1
10
CRM45 (μg/ml)

FIGURE 7

Effect of ammonium chloride on the toxicity of CRM45. Cells were incubated for 12 hr at 37°C with various concentrations of CRM45 in the presence (-O-) or absence (-●-) of 5 mM ammonium chloride. After incubation, the rate of protein synthesis was determined as described in MATERIALS AND METHODS.

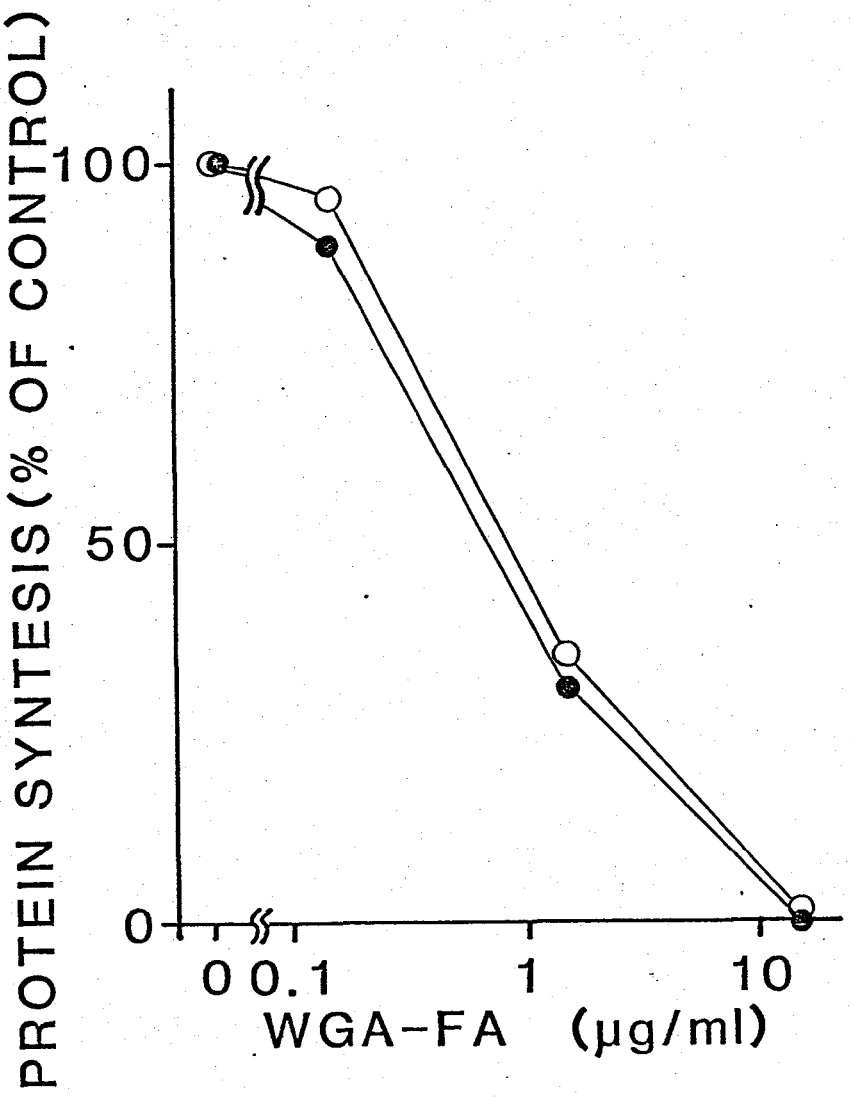
PROTEIN SYNTESIS (% OF CONTROL)
100
50
0
0 0.1
1
10
WGA-FA (μg/ml)

FIGURE 8

Effect of HVJ on the toxicity of hybrid toxin for L cells. L cells were incubated with various concentrations of hybrid toxin consisting of WGA and fragment A of diphtheria toxin at 4°C for 30 min. Then the cultures were incubated with HVJ (2,000 HAU per ml of MEM) or MEM at 4°C for 30 min. Medium was replaced with fresh medium, and the cultures were incubated at 37°C for 12 hr. After incubation, the rate of protein synthesis was determined as described in MATERIALS AND METHODS.

PART 3

SUMMARY

Fragment A of diphtheria toxin was trapped in liposomes prepared from lecithin-cholesterol (naked liposomes) or the same lipids plus either the hemagglutinating and neuraminidase (HN) or fusion (F) glycoprotein of Sendai virus (HVJ). These liposomes do not kill normal cultured cells. When such liposomes were added to mixed cultures of subacute sclerosing panencephalitis virus-infected cells (SSPE cells) and human embryonic lung (HEL) cells, the SSPE cells were selectively killed. Fragment-A containing liposomes with HN protein killed more effectively than naked liposomes containing fragment A. The mixed culture could therefore be "cured" by elimination of SSPE cells. Fragment A-containing liposomes with F protein had only about the same cytotoxicity for SSPE cells as naked liposomes containing fragment A. Monoclonal antibody HN-1, which blocks the hemagglutinating and neuraminidase activities of HN protein, reduced the cytotoxicity of fragment A-containing liposomes with HN protein to the value for naked liposomes containing fragment A. Monoclonal antibody HN-2, which does not block the hemagglutinating or neuraminidase activity but does inhibit HVJ-mediated cell fusion, partially blocked the enhanced cytotoxicity due to HN protein. Enhanced cytotoxicity due to association of HN protein with liposomes containing fragment A was also observed for SSPE cells treated with neuraminidase. With neuraminidase-treated SSPE cells the enhanced cytotoxicity was completely abolished by antibody HN-2. The implications of these results for the functions of HN protein and possible reasons for the selective killing of SSPE cells are discussed.

INTRODUCTION

Subacute sclerosing panencephalitis (SSPE) is a slowly progressive and highly lethal disease of the central nervous system in children and young adults (45) and is caused by persistent infection of a defective measles virus (18). The virus is presumably defective in synthesis of matrix (M) protein (12, 13, 23, 57). The Biken strain of SSPE virus is a defective mutant of measles virus, which was isolated from brain cells of a SSPE patient and has been propagated in human embryonic lung (HEL) cells (53). As the HEL cells infected with the Biken strain do not produce infectious cell-free virus, the strain can be successfully cultured and passaged only by cell-to-cell infection.

Using the Biken strain, Ueda et al. (54) showed that liposomes containing fragment A of diphtheria toxin were more toxic for SSPE virus-infected cells (SSPE cells) than for normal cells. Fragment A of diphtheria toxin was not toxic when added to the culture medium (50), but was toxic when only a single molecule was introduced into the cell cytoplasm (58) (see GENERAL INTRODUCTION). The greater toxicity of liposomes containing fragment A for SSPE cells indicates that fragment A inside vesicles was introduced more efficiently into the cytoplasm of SSPE cells than into that of normal cells.

Uchida et al. (51) have previously shown that artificially prepared liposomes associated with HVJ glycoproteins (F and HN) can effectively introduce proteins trapped within the liposomes into cells. The viral glycoproteins increase the efficiency of fusion between liposomes and cell membranes, and thus facilitate the transfer of foreign proteins into the cytoplasm. Although liposomes without HVJ glycoproteins (naked liposomes) readily bind to the cell surface, most of them apparently fail to fuse with the cell membrane (38), and the efficiency of transfer of proteins from the liposomes into the cytoplasm is low. In the case of SSPE cells,

liposomes associated with HVJ glycoproteins and containing fragment A kill both SSPE cells and HEL cells similarly, but naked liposomes containing fragment A kill SSPE cells selectively (*54*). This greater toxicity of naked liposomes containing fragment A to SSPE cells is, thus, due to some change in the cell membrane of SSPE cells.

In this paper, I report that fragment A containing-liposomes associated with HN protein kill SSPE cells more effectively than fragment A-containing liposomes without HVJ glycoproteins. The role of HN protein in increasing the selective lethal activity of liposomes for SSPE cells is discussed in light of the effects of monoclonal antibodies against HN protein on the lethal activity.

RESULTS

Effect of Fragment A-containing liposomes with HN or F protein on SSPE cells

We prepared fragment A-containing liposomes associated with HN protein or F protein. Although such fragment A-containing liposomes with only one of the HVJ glycoproteins are not toxic for normal cultured cells (33) (see part 1), after addition of these liposomes to mixed cultures of SSPE cells and normal human embryonic lung (HEL) cells, SSPE cells were selectively killed and eliminated from the cultures. The regions where SSPE cells had been were then occupied by normal cells, and thus the cultures appeared to be "cured" by the growth of normal cells in the presence of the liposomes. When the cured cell sheet was stained with fluorescent anti-measles virus antibody, no stained areas were observed, while many fluorescent regions were seen in mixed cultures not treated with liposomes containing fragment A. In mixed cultures of SSPE and HEL cells, the mutant measles virus within the SSPE cells infected adjacent HEL cells by cell fusion. Occasional lysis of giant cells formed plaques in the cell sheet. In untreated cultures this process continued until the whole cell sheet was destroyed. In cultures treated with fragment A-containing liposomes, the giant cells were eliminated and a confluent monolayer was formed.

The cytotoxicity of fragment A-containing liposomes with HN or F proteins was compared with that of naked liposomes containing fragment A. We determined the number of plaque-forming units (PFU) in mixed cultures of SSPE and HEL cells after exposure of the cultures to various amounts of each liposome preparation (Table 5). As the table 5 shows, the lethal effect of fragment A-containing liposomes with HN protein on SSPE cells was greater than that of fragment A-containing liposomes with F protein, which had about the same activity as naked liposomes containing fragment A. None of liposome preparations used in these

experiments decreased the number of viable cells in uninfected cultures of HEL cells, CV-1 cells, L cells, or FL cells.

HN protein increases selective toxicity of liposomes containing fragment A for SSPE cells

As HN protein increased the selective killing of SSPE cells by fragment A-containing liposomes, we made additional comparisons of the cytotoxicity of liposomes with HN protein and naked liposomes. The effect of each type of liposome on the plaque-forming ability of mixed cultures of SSPE and HEL cells was determined after exposure of mixed cultures to liposomes for one hour or 48 hours. When cells were incubated with liposomes for one hour, the number of PFU was reduced to 50% of the control value by treatment with HN protein-liposomes containing 0.7 ng fragment A, while with a similar preparation of naked liposomes treatment with liposomes containing 12 ng of fragment A was required to obtain the same effect. The cytotoxicity of the liposomes with HN protein was 10-20 times greater than that of naked liposomes (Fig.9A). When the mixed cultures were incubated with liposomes for 48 hours, liposomes with HN protein that contained 0.4 ng fragment A reduced the PFU to 10% of the control; with the naked liposome preparation, liposomes containing 80 ng of fragment A were required (Fig.9B). Depending on the level of inhibition used for comparison, the cytotoxicity for SSPE cells of fragment A-containing liposomes with HN protein was 50-200 times greater than that of naked liposomes. With longer exposure of cells to liposomes the effect of HN protein on the selective killing of SSPE cells increased.

Fragment A-containing liposomes associated with less HN protein (about 150 HAU/ml rather than 5,000 HAU/ml) were prepared. The toxicity of the liposomes with 150 HAU/ml was only 5-7 times that of naked liposomes containing the same

amount of fragment A, even after 16 hours incubation of the cultures with liposomes (data not shown). The cytotoxicity of liposomes containing fragment A for SSPE cells thus depends on the amount of HN protein bound to the liposomes.

Effect of two kinds of monoclonal antibodies against HN protein on killing of SSPE cells by fragment A-containing liposomes with HN protein

Recently, two kinds of monoclonal antibodies against HN protein of HVJ (HN-1 and HN-2) have been isolated (30). HN-1 inhibits neuraminidase activity, hemagglutinating activity and fusion activity of HVJ particles at a molar ratio of antibody to HN protein of about 1. HN-2 does not inhibit the neuraminidase or hemagglutinating activity even at a molar ratio of antibody to HN protein greater than 8, but nevertheless inhibits fusion between viral envelopes and cell membranes and cell-cell fusion mediated by HVJ. Monoclonal antibodies and fragment A-containing liposomes with HN protein were mixed at a molar ratio of antibody to HN protein of approximately 4, incubated at 37°C, diluted, and then added to mixed cultures of SSPE and HEL cells. As can be seen in Fig.10A, antibody HN-1 decreased the cytotoxicity for SSPE cells of fragment A-containing liposomes with HN protein about 20 fold, to almost the same value as naked liposomes containing fragment A. Antibody HN-2 also decreased the cytotoxicity of the liposomes with HN protein, but only by 5-7 fold.

When mixed cultures of SSPE and HEL cells were treated with neuraminidase, the cells could not be aggregated by HVJ, but the viability of normal cells and the plaque forming ability of the SSPE cells were not affected. Fragment A-containing liposomes with HN protein killed the neuraminidase-treated SSPE cells 5-7 times more effectively than naked liposomes containing fragment A. The cytotoxicity of the liposomes with HN protein for the neuraminidase-treated SSPE cells was reduced to the same level as that of naked liposomes by either HN-1 or HN-2 at a

molar ratio of antibody to HN protein of 4 (Fig.10B). Thus with neuraminidase-treated cells, the enhancement of the killing of SSPE cells by HN protein was completely blocked by either antibody.

To examine in more detail the effect of the monoclonal antibodies on the cytotoxicity of fragment A-containing liposomes with HN protein, various amounts of antibody and a fixed amount of fragment A-containing liposomes with HN protein (2,500 HAU/ml) were mixed and the cytotoxicity of the mixtures for SSPE cells determined (Fig.11). Monoclonal antibody HN-1 reduced the cytotoxicity for SSPE cells of fragment A-containing liposomes with HN protein to about the same level as naked liposomes at a molar ratio of antibody to HN protein of about 4. With antibody HN-2, the blocking activity reached a plateau at a molar ratio of antibody to HN protein of 4 but the maximum blocking activity was 30-35% of that of HN-1. Normal mouse IgG did not affect the cytotoxicity of the liposomes. From these results it is clear that HN protein associated with the liposomes plays an important role in increasing the cytotoxicity for SSPE cells.

DISCUSSION

Fragment A-containing liposomes with HN protein have a potent selective toxicity for SSPE cells. Uninfected cultured cells are not killed by these liposomes. This suggests that liposomes with HN protein that bind to SSPE cells can introduce fragment A into the cytoplasm efficiently, but liposomes that bind to normal cells can not. So, this difference in toxicity for SSPE cells and normal cells is due to the changes that have occurred in the membrane of SSPE cells. Measles virus components, mainly F protein, are present on the surface of SSPE cells (3). However, the measles F protein does not appear to be responsible for the increased toxicity of liposomes to SSPE cells, as our preliminary experiments showed that the toxicity of the liposomes was not affected by anti-measles virus antibody containing anti-F antibody (data not shown). As shown in chapter 2, the cell membrane of HVJ-treated cells was changed so as to readily fuse with liposomes. Both HVJ and measles virus belong to the paramyxovirus group, and have cell fusion activity and hemolytic activity (34). The cell membrane of SSPE cells may have been changed to allow fusion with lipid vesicles and with other cells as the result of the persistent infection with measles virus.

Holmgren et al. (17) and Markwell et al. (28) were reported that the structure NeuAcα2→8NeuAcα2→3Galβ1→3GalNAc→ is the recognition-specific structure of the receptor for HVJ which is present in both gangliosides and glycoproteins on the cell surface. The effect of HN protein on the toxicity of liposomes to SSPE cells seems to be due to the binding activity of HN-associated liposomes to receptors for HVJ on the cell surface, especially to gangliosides on lipid bilayer. By binding to gangliosides, liposomes might be able to reach easily the lipid bilayer of cell membrane.

From the difference in the effects of the monoclonal antibodies against HN

protein, HN-1 and HN-2, both on the activity of HVJ and on the cytotoxicity of fragment A-containing liposomes with HN protein for SSPE cells, it appears likely that HN protein has another activity in addition to the neuraminidase activity and hemagglutinating activity. Antibody HN-2, which does not inhibit the neuraminidase or hemagglutinating activity of HN, nevertheless partially reduced the cytotoxicity of liposomes with HN protein for SSPE cells (Fig.10A), and with neuraminidase-treated SSPE cells HN-2 reduced the cytotoxicity to the same level as naked liposomes (Fig.10B). A possible explanation for these results is that antibody HN-2 blocks an interaction of HN protein with the membrane that takes place subsequent to the initial binding of HN protein to sialic acid residues. This second interaction could be for example binding of HN protein to a different, unidentified cell surface component. Antibody HN-1 decreased the cytotoxicity of fragment A-containing liposomes with HN protein to the same values as naked liposomes. Antibody HN-1 thus inhibits not only the initial binding to sialic acid residues but also the second interaction, perhaps by masking or disturbing the conformation of the second active site of HN protein. This second active site might interact with the structure Galβ1$\rightarrow$3GalNAc of HVJ receptor, because this structure, as well as sialic acid residues, is also important for HVJ binding activity (17, 28).

In the study of cell-cell fusion (fusion between cell membranes) mediated by HVJ, the greatest difficulty is that changes in cell membrane which seem to cause cell-cell fusion succeedingly are observed only for a short time just after envelope fusion (fusion between viral envelope and cell membrane), which was clearly separatable from cell-cell fusion (31). The changes in the cell membranes of HVJ-treated cells and SSPE cells seem very similar, so investigation of changes in SSPE cell membranes may provide a clue to the process of cell-cell fusion mediated by HVJ. Furthermore, the killing of SSPE cells by fragment A-containing liposomes

provides a system for the study of the interaction of HN protein and the cell surface. In addition, the selective killing of virus-infected cells may be of clinical importance.

Treatment	ng fragment A per ml of liposomes suspension	uninfected HEL viable cells (x 10^4/dish)	PFU of SSPE per dish number	(%)
control	-	60.3	5700	(100)
Frag. A-containing liposomes with F protein*	80	56.7	1075	(18.9)
	20	67.0	2150	(37.7)
	5	62.4	3825	(67.1)
	1.25	68.4	4500	(79.0)
Frag. A-containing liposomes with HN protein**	50	60.9	35	(0.6)
	12.5	59.7	493	(8.6)
	3.1	61.1	1325	(23.2)
	0.8	66.3	1875	(32.9)
Frag. A-containing liposomes without F or HN protein***	120	60.9	1450	(25.5)
	30	57.6	2175	(38.2)
	7.5	69.3	2975	(52.2)
	1.9	67.8	4375	(76.8)

Initial concentration per ml:
* 0.075 OD540nm, 80 ng fragment A.
** 0.04 OD540nm, 50 ng fragment A, 750 HAU
*** 0.126 OD540nm, 120 ng fragment A.

TABLE 5

Cytotoxicity of various liposomes for SSPE cells and uninfected HEL cells. Various types of liposomes containing fragment A were added to the mixed culture of SSPE cells and HEL cells. Infectivity of the liposome-treated cultures was measured by an infectious center assay and expressed as plaque forming units (PFU) per dish as described in MATERIALS AND METHODS.

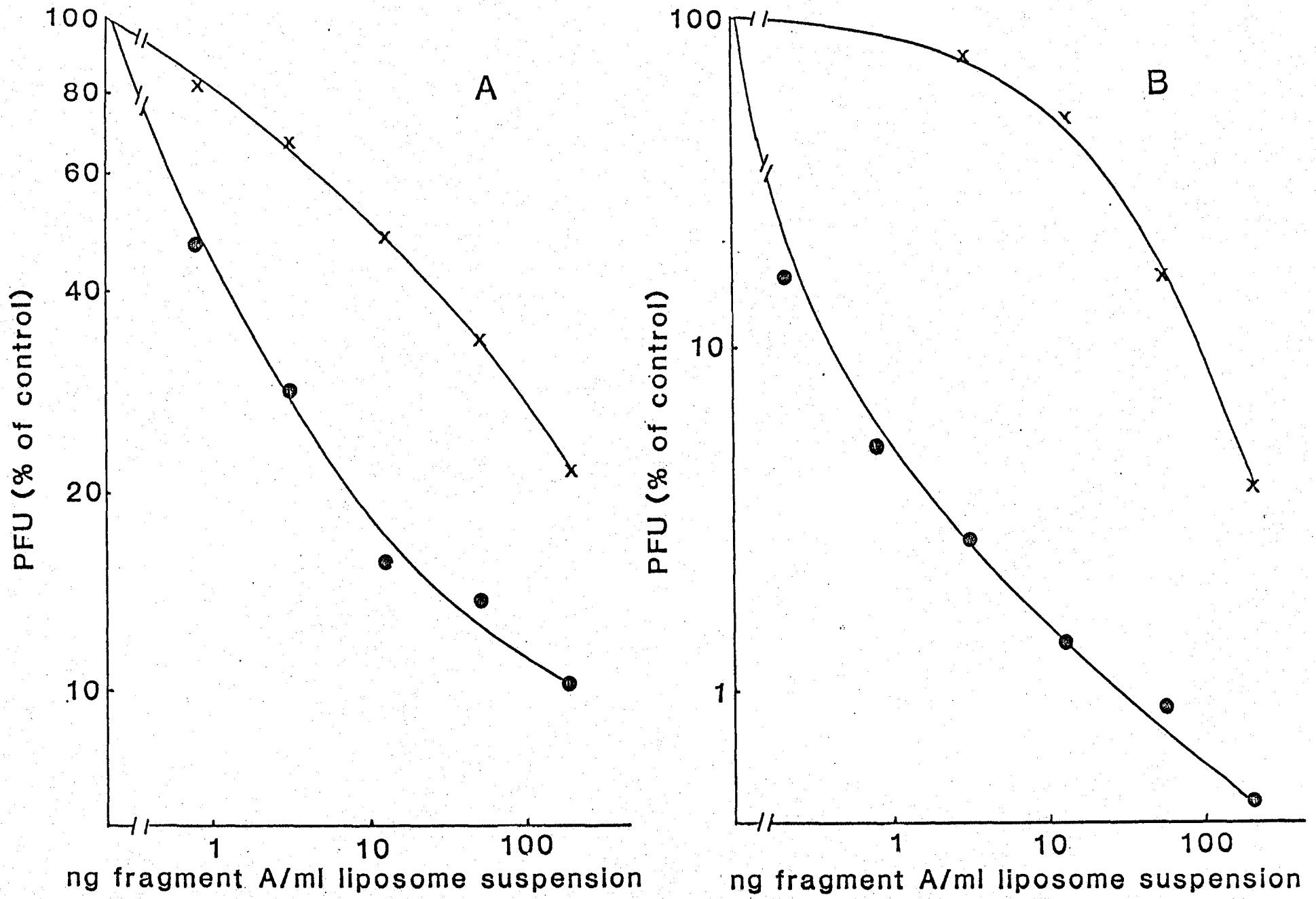
A
B
100
80
60
40
20
10
1
PFU (% of control)
1
10
100
ng fragment A/ml liposome suspension

FIGURE 9

Cytotoxicity of fragment A-containing liposomes with HN protein compared to that of naked liposomes containing fragment A. Fragment A-containing liposomes with HN protein (one ml of the original suspension contained 200 ng fragment A, 5,000 HAU/ml, and had an OD_{540} of 0.2) and naked liposomes (OD_{540} = 0.24, 200 ng fragment A/ml) were diluted and added to the mixed cultures of SSPE and HEL cells. (A) The mixed cultures were treated with liposomes for one hour at 37°C, and then washed to remove free liposomes and further incubated at 37°C for 2 days. (B) The mixed cultures were exposed to the liposomes for 2 days at 37°C. PFU were determined as described in MATERIALS AND METHODS. The number of PFU in mixed cultures incubated in parallel without liposomes was taken as 100%. The PFU in control cultures were 1,600 in experiment A and 3,350 in experiment B. Fragment A-containing liposomes with HN protein (-●-); naked liposomes (-✕-).

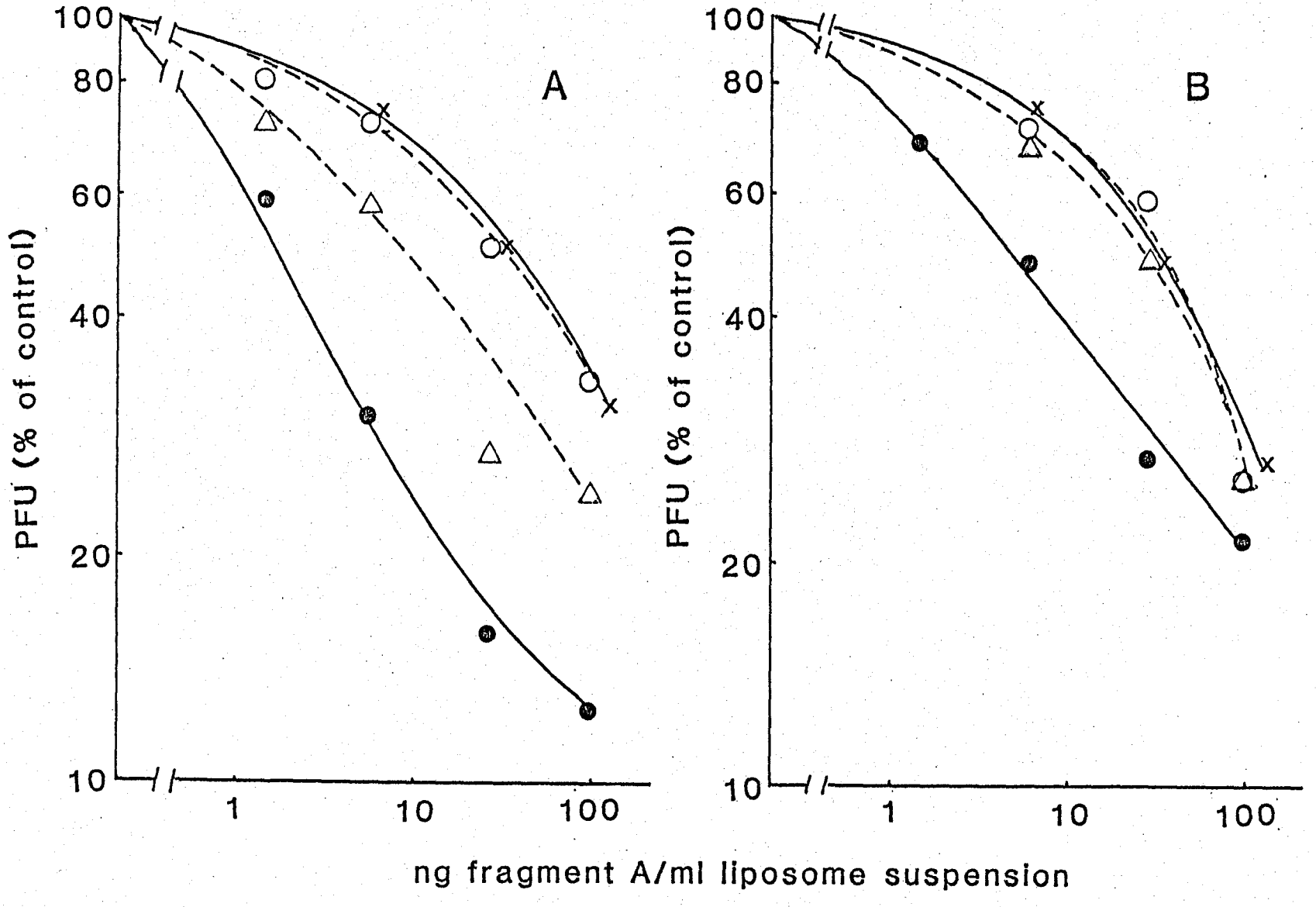
A
B
100
80
60
40
20
10
PFU (% of control)
1
10
100
ng fragment A/ml liposome suspension

FIGURE 10

Effect of antibody against HN protein on cytotoxicity of fragment A-containing liposomes with HN protein for SSPE cells and neuraminidase-treated SSPE cells. Mixed cultures of SSPE cells and HEL cells were prepared as described in MATERIALS AND METHODS. The cultures in (B) were treated with neuraminidase (6 mUnits/ml) for 1 hr at 37° C and then washed with medium. Fragment A-containing liposomes with HN protein (initial concentration OD_{540} = 0.11, 90 ng fragment A and 1,600 HAU per ml) or naked liposomes (OD_{540} = 0.23, 110 ng fragment A per ml) were mixed with 150 μg monoclonal antibody HN-1 or HN-2, or 150 μg pre-immune mouse IgG, and the mixtures were added to the cultures. After incubation at 37 °C for 2 hr, the cultures were washed with HBSS to remove free liposomes, fed with 2.5 ml medium 199/MEM with 5% fetal bovine serum, and incubated for 2 days. PFU for the controls in A and B were 3,075 and 3,470, respectively. (-●-) Fragment A-containing liposomes with HN protein plus preimmune IgG; (-✕-) Naked liposomes plus pre-immune IgG; (-○-) Fragment A-containing liposomes with HN protein plus antibody HN-1; (-△-) Fragment A-containing liposome with HN protein plus antibody HN-2.

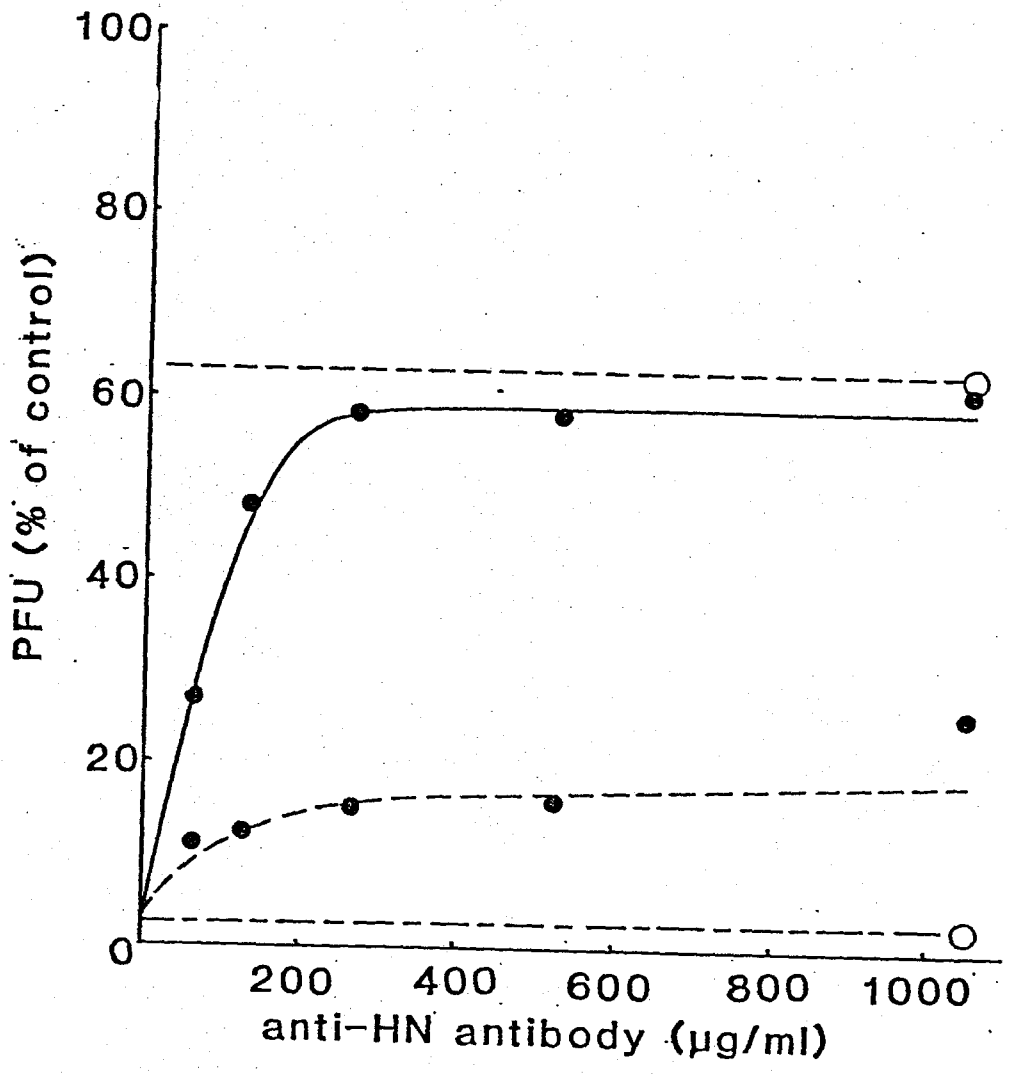
100
80
60
40
20
0
PFU (% of control)
200
400
600
800
1000
anti-HN antibody (μg/ml)

FIGURE 11

Effect of various amount of monoclonal antibodies HN-1 and HN-2 on the cytotoxicity for SSPE cells of a fixed amount of fragment A-containing liposomes with HN protein. Various amounts of monoclonal antibody (32-1050 μg) were added to fragment A-containing liposomes with HN protein (2,500 HAU and 500 ng fragment A per ml, OD_{540} = 0.03) or naked liposomes (500 ng fragment A per ml). The mixtures were incubated at 37°C for 30 min and then added to mixed cultures of SSPE and HEL cells. After incubation at 37°C for 2 hr the cultures were washed with HBSS to remove free liposomes and then cultured for 2 days. PFU were determined as described in MATERIALS AND METHODS. (-○-) Naked liposomes containing fragment A plus 1,050 μg pre-immune mouse IgG; (-●-) fragment A containing liposomes with HN protein plus antibody HN-1; (-●-) fragment A-containing liposomes with HN protein plus antibody HN-2. In this experiment the PFU of the control was 978.

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