

# INTEGRATION OF A VIRUS MEMBRANE PROTEIN INTO THE LIPID BILAYER OF TARGET CELLS AS A PREREQUISITE FOR IMMUNE CYTOLYSIS

## Specific Cytolysis after Virosome-Target Cell Fusion

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Target cell lysis can be achieved by different specific effector mechanisms: antibody and complement (complement-dependent lysis, [CDL]<sup>1</sup>), antibody and K cells (antibody-dependent cell-mediated cytotoxicity, [ADCC]) or cytolytic T lymphocytes (CTL). Little is known about how the target antigens have to be presented on the cell membrane to allow specific target cell lysis and whether the different effector mechanisms have similar structural requirements for cytotoxicity. We have studied these questions in a model system in which purified viral spike proteins were used as target cell antigens. We were particularly interested in finding out whether it is sufficient that viral antigens are bound to cells or whether integration of the viral antigens into the lipid bilayer of the plasma membrane is a prerequisite for cytotoxicity. Our earlier data (1, 2) indicated that viral antigen integration is required for lysis by H-2-restricted CTL. In this report we studied this question with respect to cytotoxic events involving antibodies.

We have used Semliki Forest virus (SFV), a membrane virus which is assembled by a budding process. SFV has only one spike protein, composed of three polypeptide chains, which is inserted into the plasma membrane at the budding site. This spike protein can be prepared from purified SFV in three different physical forms: (a) as a monomer bound to detergent (3), (b) as a practically lipid- and detergent-free protein micelle in an octameric form (29S complex) (4), or (c) reconstituted into detergent-free lipid vesicles—so-called virosomes (5).

The micelles and most likely also the virosomes attach to receptors on the cell

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<sup>1</sup> Abbreviations used in this paper: ADCC, antibody-dependent cell-mediated cytotoxicity; BPL,  $\beta$ -propiolactone; BPL-SV, BPL-treated Sendai virus; C', complement; CDL, complement-dependent lysis; complete RPMI-1640 medium, RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50  $\mu$ g streptomycin, and 10 mM Hepes, pH 7.3; CTL, cytolytic T lymphocytes; F, fusion protein; FITC, fluorescein isothiocyanate; HN, hemagglutinin-neuraminidase; IIF, indirect immunofluorescence; MES, 2-(*N*-morpholino)ethane sulfonic acid; pfu, plaque-forming unit; PBS, phosphate-buffered saline; Rab  $\alpha$ -SFV, rabbit anti-Semliki Forest virus; SFV, Semliki Forest virus; SV, Sendai virus; Tryp-SV, trypsin-treated SV; Staph-SV, SV treated with V8 protease of *Staphylococcus aureus*.

surface, which are shown to be the histocompatibility antigens (6). The requirements of viral antigen expression on tumor target cells for immune cytotoxicity were studied by comparing target cells prepared in three different ways: (a) virus-infected cells, where the antigens are integrated into the cell membrane from within, (b) cells bearing the viral antigens attached to receptors on the cell surface, and (c) cells which have the viral antigens integrated into the cell membrane from the outside by fusion of target cells with virosomes. Because the virosomes did not fuse spontaneously with the cell membrane, we used Sendai virus to facilitate the fusion (Fig. 1).

Immune cytotoxicity of target cells was measured by the  $^{51}\text{Cr}$ -release technique after incubation with either anti-viral antibodies and complement (CDL) or anti-virus antibody and K cells (ADCC). In addition, in selected experiments anti-virus CTL were also employed. We will show that viral antigen binding to cells is not sufficient and that integration of viral glycoproteins is a prerequisite for anti-viral immune cytotoxicity in all three assays investigated.

### Materials and Methods

*Virus and Preparation of Virus Antigen.* A prototype strain of SFV was propagated in monolayer cell cultures of BHK-21 cells. The culture procedure, virus purification, and purity controls were performed as described by Kääriäinen et al. (7). The lipid-free octamer membrane protein complexes (protein micelles), which have an  $s$  of 29S (therefore also referred to as 29S complexes), were prepared by solubilization of whole virus with Triton X-100 followed by centrifugation through a top-layer zone of Triton X-100 into a 20–50% sucrose gradient according to Helenius and Bonsdorff (4). Liposomes with inserted virus spike proteins (virosomes) were prepared with the octylglycoside dialysis method of Helenius et al. (5). The protein content of the virus preparations was determined by the Lowry method (8) with 3.5 mM sodium dodecyl sulfate in the reaction mixture.

Sendai virus (SV) was grown in the allantoic cavity of 10-d-old embryonated chicken eggs. The virus was concentrated from the allantoic fluid by centrifugation at 120,000  $g$  for 1 h at 4°C. It was resuspended in phosphate-buffered saline (PBS) and purified by density-gradient centrifugation (20–50% sucrose (wt/vol)) in PBS at 100,000  $g$  for 2 h at 4°C. Purified SV was inactivated with  $\beta$ -propiolactone (BPL) (9). Proteolytic digestion of BPL-treated SV (BPL-SV) was achieved either by trypsin, which cleaves the fusion protein (F) (10), or by V8 protease from *Staphylococcus aureus* which removes the hemagglutinin-neuraminidase (HN) glycoprotein (2).

*Antisera.* Mouse anti-SFV serum was prepared by inoculating  $10^4$  plaque-forming units (pfu) of SFV A774 strain (11) i.p. into BALB/c mice. The mice were bled 17 d after infection (BALB/c  $\alpha$ -SFV). Rabbit anti-SFV serum (Rab  $\alpha$ -SFV) was prepared by immunizing rabbits with purified spike proteins of SFV in the octamer complex (protein micelles) form. It was kindly provided by Dr. K. Simons, European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany. Mouse anti-SV serum was raised in DBA/2 mice by three injections of 100 hemagglutinating units i.p. at weekly intervals. The mice were bled 15 d after the last injection (DBA/2  $\alpha$ -SV). Rabbit anti-SV(F) serum was an antiserum against purified SV F. It was kindly provided by Dr. M.-J. Gething, Imperial Cancer Research Fund, London, England. Anti-H-2<sup>d</sup> serum was prepared by 6–10 repeated immunizations of CBA/J mice with  $10^7$  spleen cells from a BALB/c mouse (CBA/J  $\alpha$ -BALB/c) i.p. Anti-non-H-2 serum was obtained by grafting DBA/2 skin onto B10.D2 mice followed 22 and 32 d later by a challenge with  $10^7$  DBA/2 spleen cells i.p. (B10.D2  $\alpha$ -DBA/2). It was a gift from Dr. Berenice Kindred, Heidelberg, Federal Republic of Germany.

*Complement.* Normal rabbit serum, absorbed with mouse spleen cells, served as a source of complement when using P815 cells as target cells. Normal guinea pig serum (Behring Werke AG, Marburg/Lahn, Federal Republic of Germany), absorbed with agarose and sheep erythrocytes, was used in CDL assays using Eb cells as target cells.

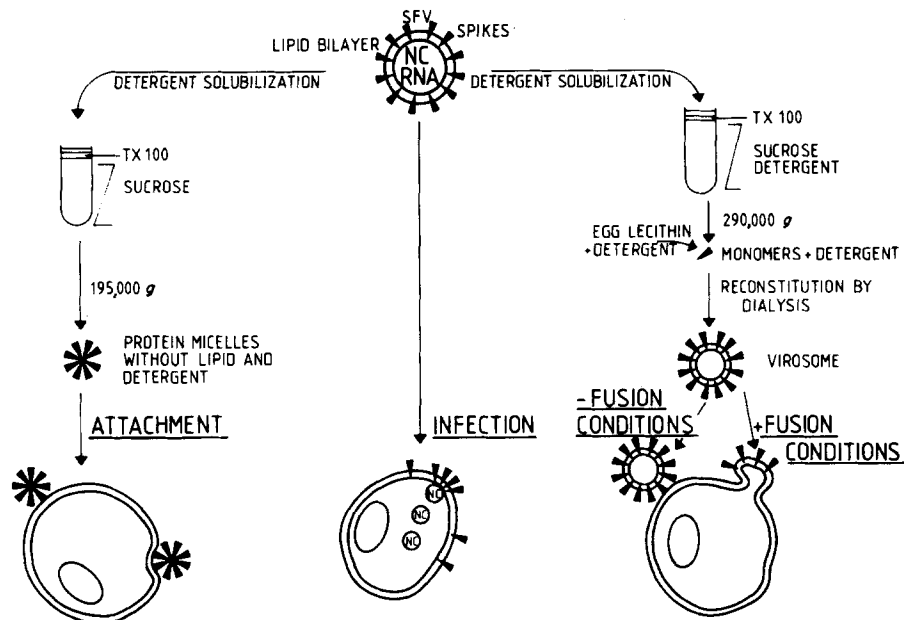


FIG. 1. Schematic drawing of the preparation of SFV antigens in different physical forms and of their use in target cell generation showing three different characteristics of antigen presentation: (a) cells expressing the viral antigens bound to receptors on the cell surface by attachment of SFV-29S complexes or by incubation with SFV-virosomes, (b) cells expressing the viral antigens integrated in the cell membrane from within by SFV infection, and (c) cells expressing the viral antigens integrated in the cell membrane from without by incubation with virosomes from SFV under fusion conditions. TX 100, Triton X-100.

#### Effector Cells

**K CELLS.** Human peripheral blood lymphocytes obtained by the Isopaque-Ficoll (Pharmacia Fine Chemicals Inc., Div. of Pharmacia, Inc., Piscataway, N. J.) separation method (12), were used as effector cells in ADCC assays.

**CTL.** Anti-SV-specific CTL were generated as described (13). They were secondary CTL from DBA/2 mice sensitized *in vivo* and restimulated *in vitro*.

**Target Cells.** The tumor cell lines P815 (a mastocytoma cell) and Eb (a lymphoma cell) (14) are both derived from DBA/2 (H-2<sup>d</sup>) mice. They were grown in suspension culture at 37°C in a 5% CO<sub>2</sub> atmosphere using RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin 50 µg/ml streptomycin, and 10 mM HEPES, pH 7.3 (complete RPMI-1640 medium). The cells were harvested in the exponential growth phase. They were incubated with viral antigens and labeled with 25 µCi <sup>51</sup>Cr per 10<sup>6</sup> cells at the same time (see below for incubation times). Before using the cells in a cytotoxic assay, they were washed and the viability was determined by trypan blue exclusion. Target cells carrying the virus spikes integrated into the cell membrane from within were prepared for one cycle of infection by incubation with 10–100 pfu of SFV per cell in serum-free medium for 1 h at 37°C in a total vol of 200 µl. Thereafter, 1 ml of complete RPMI-1640 medium was added per 10<sup>6</sup> cells and they were further incubated for 3 h. 29S complexes from SFV were bound to the cell surface by incubation of 5–10 µg of the micelles per 10<sup>6</sup> cells in RPMI-1640 medium buffered to pH 7.0 or to pH 6.5 with 2-(N-morpholino)ethane sulfonic acid (MES). Virosomes were bound to the cell surface by incubation of 2–5 µg virus protein with the cells under similar conditions as for the 29S complexes. Integration of SFV membrane protein into the cell membrane from outside was achieved by incubation of cells with 5 µg virosomes per 10<sup>6</sup> cells and with SV with active fusion activity (amounts specified in legends to figures) for 1–4 h at 37°C in complete RPMI-1640 medium. 29S complexes (10 µg/10<sup>6</sup> cells) were also incubated

with SV as described for virosomes. Further controls included incubation of cells with virosomes and SV with inactivated HN or F.

#### Assays

**INDIRECT IMMUNOFLUORESCENCE (IIF).** IIF was performed as described by Möller (15).  $10^6$  cells in  $40 \mu\text{l}$  medium and  $10 \mu\text{l}$  1:10-diluted Rab  $\alpha$ -SFV serum were incubated for 30 min on ice. The cells were washed twice and suspended in  $50 \mu\text{l}$  medium plus  $50 \mu\text{l}$  1:50-diluted fluorescein isothiocyanate (FITC)-conjugated pig anti-rabbit Ig serum (Nordic Immunological Laboratories, Tilburg, The Netherlands) and incubated further on ice for 30 min. The cells were washed twice in PBS, pH 7.4, and fixed in  $50 \mu\text{l}$  of 1.8% paraformaldehyde at room temperature for 10 min. Thereafter, the cells were washed again in PBS and mounted on glass slides. In the case where the cells were coated with virosomes or micelles, the medium used was buffered to pH 6.5 with MES except for the mounting buffer, which had a pH of 7.4. The above antisera were always preabsorbed with the same cells on which they were used.

**ANTIBODY ABSORPTION.** 1 ml of antiserum was preincubated with  $1.2 \times 10^7$  of the different target cells for 30 min on ice and the remaining cytolytic activity of the antiserum was tested by CDL of SFV-infected target cells.

**COMPLEMENT CONSUMPTION.** The hemolytic complement activity left in the supernate of CDL was determined as described (16).

**IMMUNE CYTOLYSIS ASSAYS.** The cytolysis of target cells was assayed in a  $^{51}\text{Cr}$ -release test (17, 18), performed in round-bottom microtiter plates (Linbro Chemical Co., Hamden, Conn., model IS-MRC 96). In CDL  $2 \times 10^4$  target cells in a vol of  $25 \mu\text{l}$  were incubated with  $25 \mu\text{l}$  diluted antiserum and  $25 \mu\text{l}$  complement for 1 h at  $37^\circ\text{C}$ . After incubation,  $125 \mu\text{l}$  of cold medium was added. In ADCC  $2 \times 10^4$  target cells in a vol of  $75 \mu\text{l}$  were incubated with  $25 \mu\text{l}$  diluted antiserum and  $1 \times 10^6$  effector cells in  $100 \mu\text{l}$  for 4 h at  $37^\circ\text{C}$ . In T-cell lysis (CTL)  $1 \times 10^4$  target cells in  $100 \mu\text{l}$  of medium were incubated together with four different concentrations of effector cells for 4 h at  $37^\circ\text{C}$ . In all cytolysis assays the plates were centrifuged at the end of the incubation period for 10 min at  $375 g$ .  $100 \mu\text{l}$  samples of the supernates were removed and the radioactivity determined in a gamma counter (Wallac Ultrogramma; LKB Produkter, Bromma, Sweden). Results are given as the percentage of specific  $^{51}\text{Cr}$  release and were calculated from triplicates according to the formula:

$$\text{percentage of specific } ^{51}\text{Cr release} = \frac{\text{experimental counts per minute} - \text{low control}}{\text{high control} - \text{low control}} \times 100.$$

High controls were obtained by incubation of the target cells with detergent. The release from cells incubated without antiserum but in the presence of complement is referred to as low control in CDL. In the ADCC and T-cell lysis systems, the spontaneous release of target cells incubated without effector cells was taken as low control.

## Results

*Binding and Redistribution of SFV Spike Glycoprotein on Murine Tumor Cell Lines.* Before starting the cytolysis assays, the optimal conditions for binding of the viral antigens to the murine target cells (P815 and Eb) were determined by IIF. With 29S complexes, maximum intensity of fluorescence was obtained with  $10 \mu\text{g}$  of spike protein per  $10^6$  cells, after incubation on ice for 30 min at pH 6.5.<sup>2</sup> Fluorescence was detectable using as little as  $0.5 \mu\text{g}$  of spike protein. With virosomes a maximum intensity of fluorescence was obtained with 2–5  $\mu\text{g}$  of spike protein/ $10^6$  cells. More than 95% of the cells incubated with the 29S complexes or the virosomes showed fluorescence which under noncapping conditions had a patchy distribution (Fig. 2A and B). After infection of Eb or P815 cells with SFV, >95% of the cells were again positive in IIF (Fig. 2C). No fluorescence was observed with control cells without viral antigens.

Redistribution of viral antigens on the tumor cell membranes was investigated after

<sup>2</sup> For pH dependence of the binding see (19).

incubation of the antibody-coated cells at 37°C. More than 90% of the cells showed cap formation after incubation with 29S complexes and rabbit anti-viral antibodies, followed by incubation for 2 h at 37°C with the FITC-conjugated pig or sheep anti-rabbit Ig (Fig. 2D). When the capped cells were again incubated with the 29S complexes and assayed in IIF with rhodamine-conjugated sheep anti-rabbit serum no fluorescence was observed on the stripped areas of the cell.

*Target Cells with Viral Antigens Attached to the Cell Membrane are Resistant to Immune Cytolysis.* P815 cells carrying viral antigens as integral membrane proteins after infection with SFV, were lysed by anti-SFV antiserum and complement (Fig. 3).<sup>3</sup> No target cell lysis, however, was seen when the tumor cells carrying SFV 29S complexes (Fig. 2A) were incubated with anti-SFV serum and complement under the same conditions (Fig. 3). In spite of their resistance to lysis, the target cells bound antibody and consumed even more complement than the virus-infected cells (Table I).

To exclude the possibility that the cells after attachment of viral antigens were generally resistant to lysis, several antisera against other cell surface determinants were tested. As illustrated in Fig. 3, an antiserum against non-H-2 membrane antigens (B10.D2  $\alpha$ -DBA/2) lysed untreated, virus-infected and 29S-coated target cells with equal efficiency. When using an antiserum against H-2 antigens, 29S-coated cells were generally lysed less efficiently than untreated or virus-infected cells. This blocking effect of H-2-specific lysis by 29S complexes was found with reproducibility in 10 out of 12 experiments and ranged between 20 and 70% inhibition.

Analogous experiments were performed with a different immune cytotoxicity technique, ADCC, in which K cells are added to the system instead of complement. The results obtained in ADCC were very similar to those obtained in CDL (Fig. 4). Thus, the inability of anti-virus antibody to lyse target cells carrying SFV-29S complexes on the surface, does not seem to be restricted to the complement system.

*Target Cells with Viral Antigens Integrated into the Cell Membrane via Fusion are Susceptible to Immune Cytolysis.* The difference in susceptibility to lysis of virus-infected cells and 29S complex-coated cells suggested that integration of viral antigens into the cell membrane may be required for immune cytotoxicity. To test this hypothesis directly, we tried to integrate the purified viral antigens from outside into the cell membrane via fusion of SFV virosomes as outlined in Fig. 1. The target cells incubated with SFV virosomes were resistant to lysis by anti-SFV antibody and complement (Figs. 3 and 5). The cells, however, became susceptible for SFV-specific lysis when together with SFV virosomes BPL-treated SV was added as a fusing reagent (Fig. 5C). Cells incubated with SFV-29S complexes remained resistant to lysis even in the presence of SV.

The same target cell preparations were also tested in the ADCC assay. The results (Fig. 6) were in every aspect analogous to those reported above for the CDL test. Thus the K cells in the presence of antibody to SFV lysed target cells incubated with virosomes and SV with fusion activity, but not cells incubated with 29S complexes and the same preparation of SV.

Further evidence for fusion of the virosomes with the cell membrane as the decisive factor which made the cells susceptible to immune lysis was obtained from control

<sup>3</sup> This lysis appears to follow the alternative pathway of complement activation ([20] and Barz et al. Manuscript in preparation.).

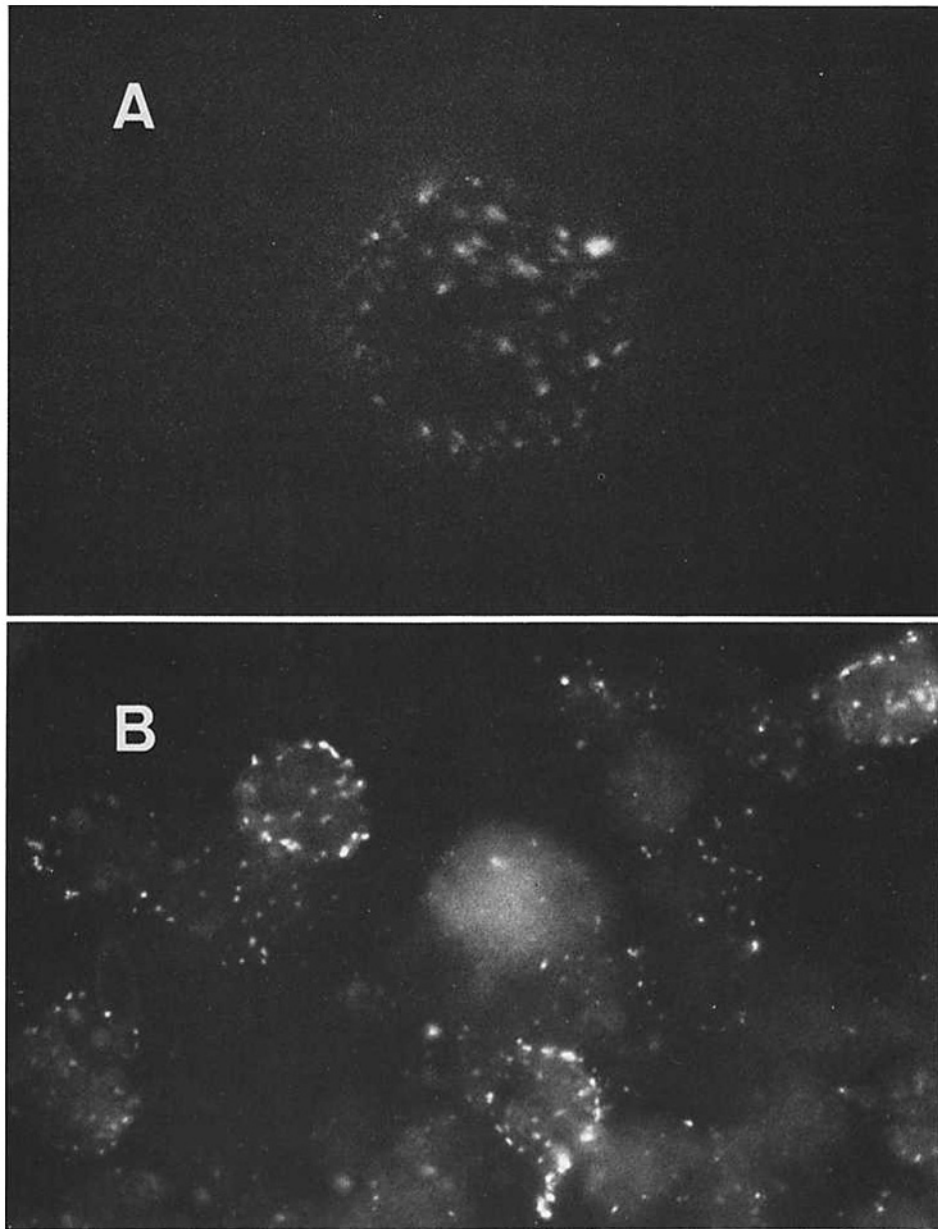


FIG. 2 A, B

FIG. 2. IIF of target cells. (A) P815 cells incubated with SFV-29S complexes.  $\times 4,000$ . (B) P815 cells incubated with SFV-virosomes.  $\times 1,500$ . (C) P815 cells infected with SFV.  $\times 4,000$ . (D) P815 cells incubated with SFV-29S complexes under capping conditions.  $\times 15,000$ .

experiments with SV preparations lacking the fusion capacity: (a) trypsin-treated SV (Tryp-SV), which has similar binding capacity as BPL-SV but carries an inactive F and (b) staphylococcus protease-treated SV (Staph-SV), which lacks the HN protein and therefore cannot bind and mediate fusion. These and the untreated SV prepa-

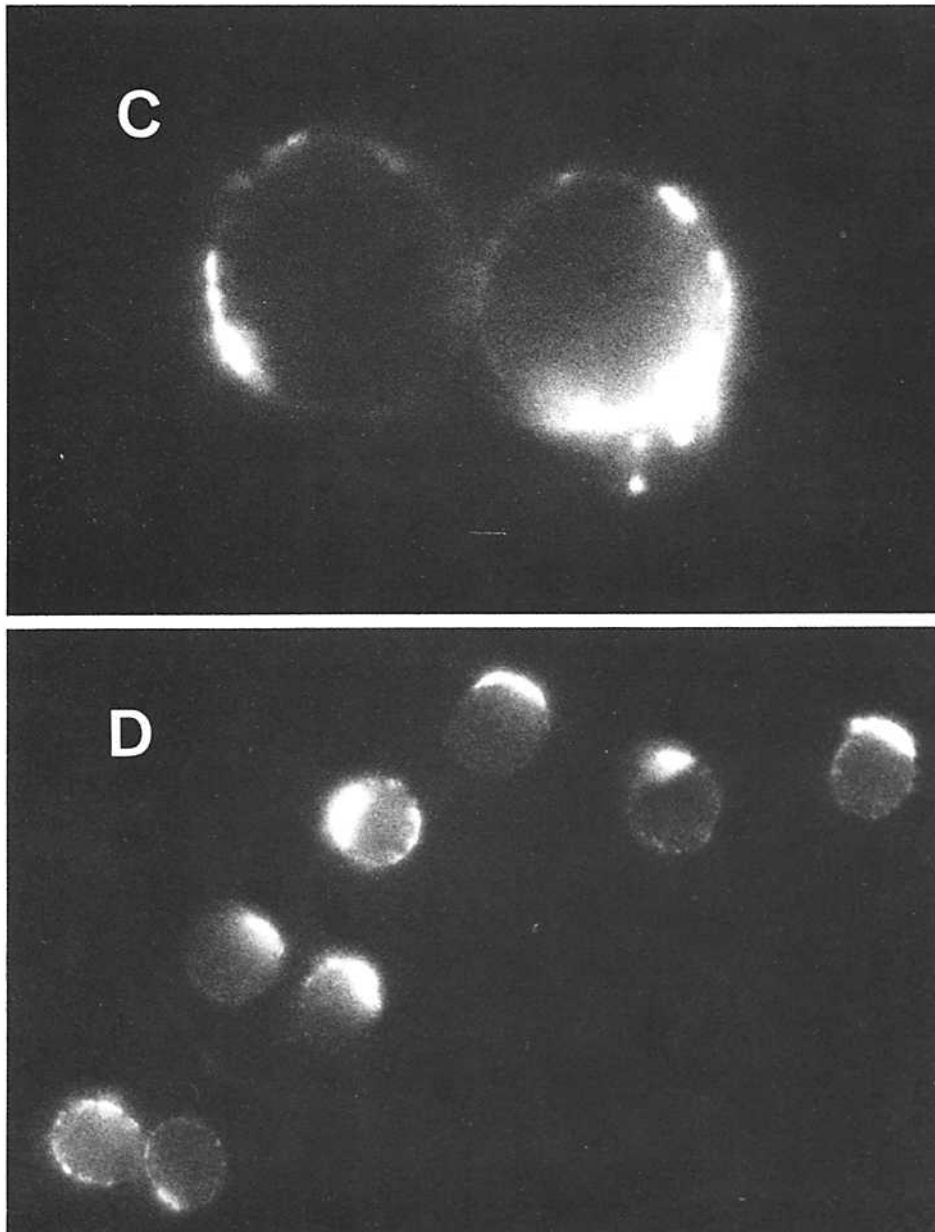


FIG. 2C, D

rations were tested not only for their capacity to mediate SFV-specific lysis but also to function as targets for SV-specific lysis (CDL, ADCC). In addition, SV-specific T-cell mediated lysis was tested which was previously found to be a very sensitive assay for the detection of membrane-integrated SV. The results (Figs. 5 and 6) showed: (a) SFV-specific lysis via virosomes (CDL, ADCC) was not facilitated by SV preparations without fusion capacity; (b) in tests for SV-specific lysis (CDL, ADCC), only the SV preparation with fusion capacity was able to generate appropriate target cells.

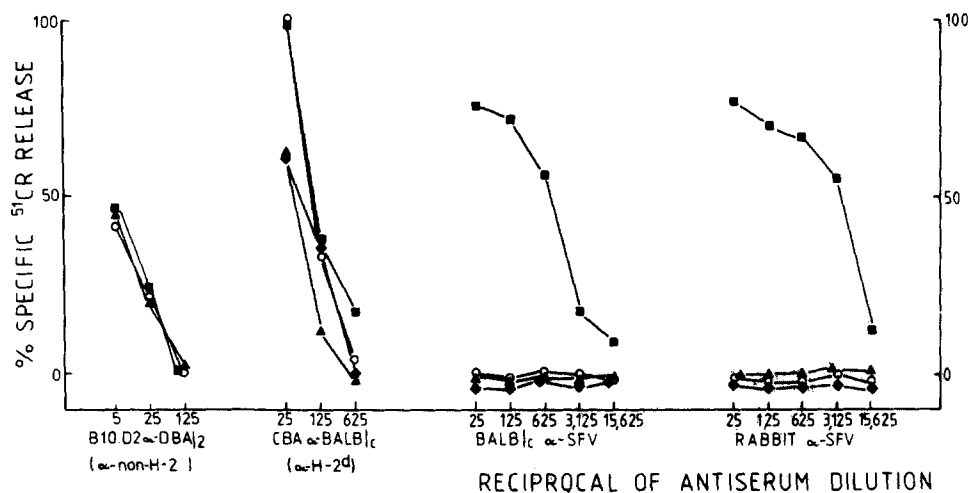


FIG. 3. CDL of P815 cells expressing SFV spike protein by virus infection or by attachment of the purified spike proteins as 29S complexes or virosomes. (○) Nontreated P815 cells; (■) SFV-infected P815 cells; (▲) P815 cells with SFV-29S complexes bound to the cell surface; (◆) P815 cells with SFV-virosomes bound to the cell surface.

TABLE I  
*Binding of Antibody and of Complement to Cells Infected with SFV or Coated with SFV-29S Complexes*

Conditions in CDL		Nontreated cells			SFV-infected cells			Cells + SFV-29S complexes		
Antiserum	C'	<sup>51</sup> Cr release*	Remain- ing com- plement activity‡	Remain- ing anti- body ac- tivity§	<sup>51</sup> Cr re- lease*	Remain- ing com- plement activity‡	Remain- ing anti- body ac- tivity§	<sup>51</sup> Cr re- lease*	Remain- ing com- plement activity‡	Remain- ing anti- body ac- tivity§
			%	%		%	%		%	%
—	—	4	7	—	6	7	—	9	9	—
—	+	5	41	—	7	32	—	10	30	—
Rabbit α-SFV	+	4	43	71	52	15	26	9	9	9
1:25										
CBA α-BALB/c	+	78	24	9	82	22	7	76	27	9
1:25										

The capacity of the different target cells to bind specific antibodies and complement (C') was compared with their ability to mediate CDL.

\* The percentage of <sup>51</sup>Cr release of the different Eb target cells in a CDL test.

‡ The percentage of remaining hemolytic activity of the complement in supernates of CDL test performed analogous to those in the <sup>51</sup>Cr-release assay but with unlabeled target cells; for each group 3-5 samples of supernate were pooled and tested against sensitized erythrocytes as described (16).

§ The percentage of remaining cytolytic activity of the antiserum when preabsorbed on either nontreated, SFV-infected, or 29S complex-coated cells (P815) and then tested in a CDL test on SFV-infected P815 target cells; for conditions of the absorption, see Material and Methods.

All the differently treated target cells could be lysed by H-2 alloantibodies and complement.

When testing the same target cell preparations for susceptibility to immune cytotoxicity by specific CTL, similar results were obtained again (Fig. 7). SV-specific CTL were shown to lyse only those tumor target cells which were sensitized with BPL-SV carrying active HN and F. When either of these proteins was destroyed or inactivated by enzymatic pretreatment, no sensitization of target cells for lysis was observed. Incubation of cells with 0.1 μg of SV was found sufficient for target cell formation in the CTL assay, but incubation with even 50 μg of Tryp-SV or Staph-SV did not make the cells susceptible to SV-specific immune lysis.



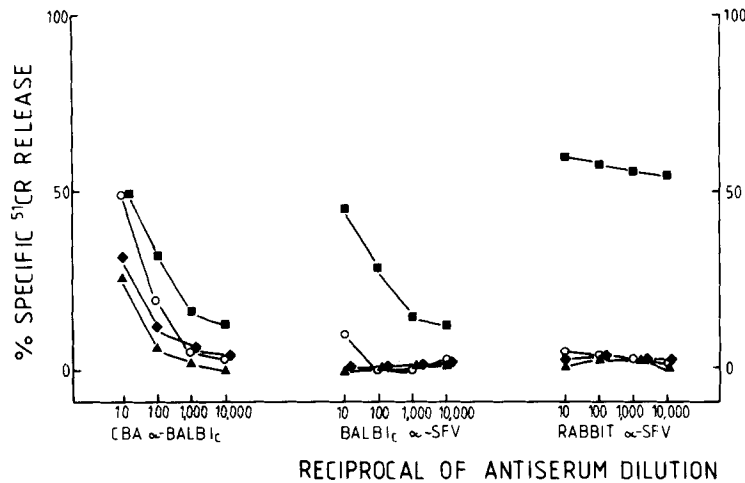


Fig. 4. ADCC of P815 cells expressing SFV spike protein by virus infection or by attachment of the purified spike proteins as 29S complexes or virosomes. (O) Nontreated P815 cells; (■) SFV-infected P815 cells; (▲) P815 cells with SFV-29S complexes bound to the cell surface; (◆) P815 cells with SFV-virosomes bound to the cell surface.

### Discussion

Integration of a virus membrane protein into the lipid bilayer of target cells has been shown to be essential for immune cytotoxicity. This came out from experiments in which the spike proteins of SFV were incorporated into target cell membranes from outside via fusion of virosomes. This kind of experiment demonstrating the successful transfer of a purified membrane protein from one particle (the virus) to another (the tumor target cell) may be of general applicability and will therefore be discussed in more detail.

The spike proteins of SFV were first isolated in detergent. Because the detergent would interfere with the stability of the target cell membranes, it was either replaced by artificial lipids whereby the SFV proteins were reconstituted into lipid vesicles (virosomes) or it was removed completely, whereby the spike proteins formed stable octameric rosettes (29S complexes). Both the virosomes and the 29S complexes had the ability to attach to the murine tumor target cells. Such cells with attached SFV antigens could not be lysed by anti-SFV antibody and complement (CDL) or by anti-SFV antibody and K cells (ADCC). When, however, fusion between the SFV-virosomes and the target cell membranes was made possible by the addition of noninfectious SV as a fusing reagent, SFV-specific lysis was observed in both the CDL and the ADCC assay. Cells with attached SFV-29S complexes remained resistant to SFV-specific lysis even in the presence of the above SV preparation.

These studies can be considered as an extension of previous liposome-cell interaction experiments which enabled the introduction of the haptenic determinant dinitrophenyl into target cell membranes (21, 22, 23). The new aspects from our system are: (a) use of a natural membrane protein (SFV) rather than a lipophilic artificial substance; (b) comparison of different physical forms of the same membrane protein; and (c) separation of the fusion process itself by using an independent and well-characterized antigenic system as mediator for fusion. Thus it could be shown that

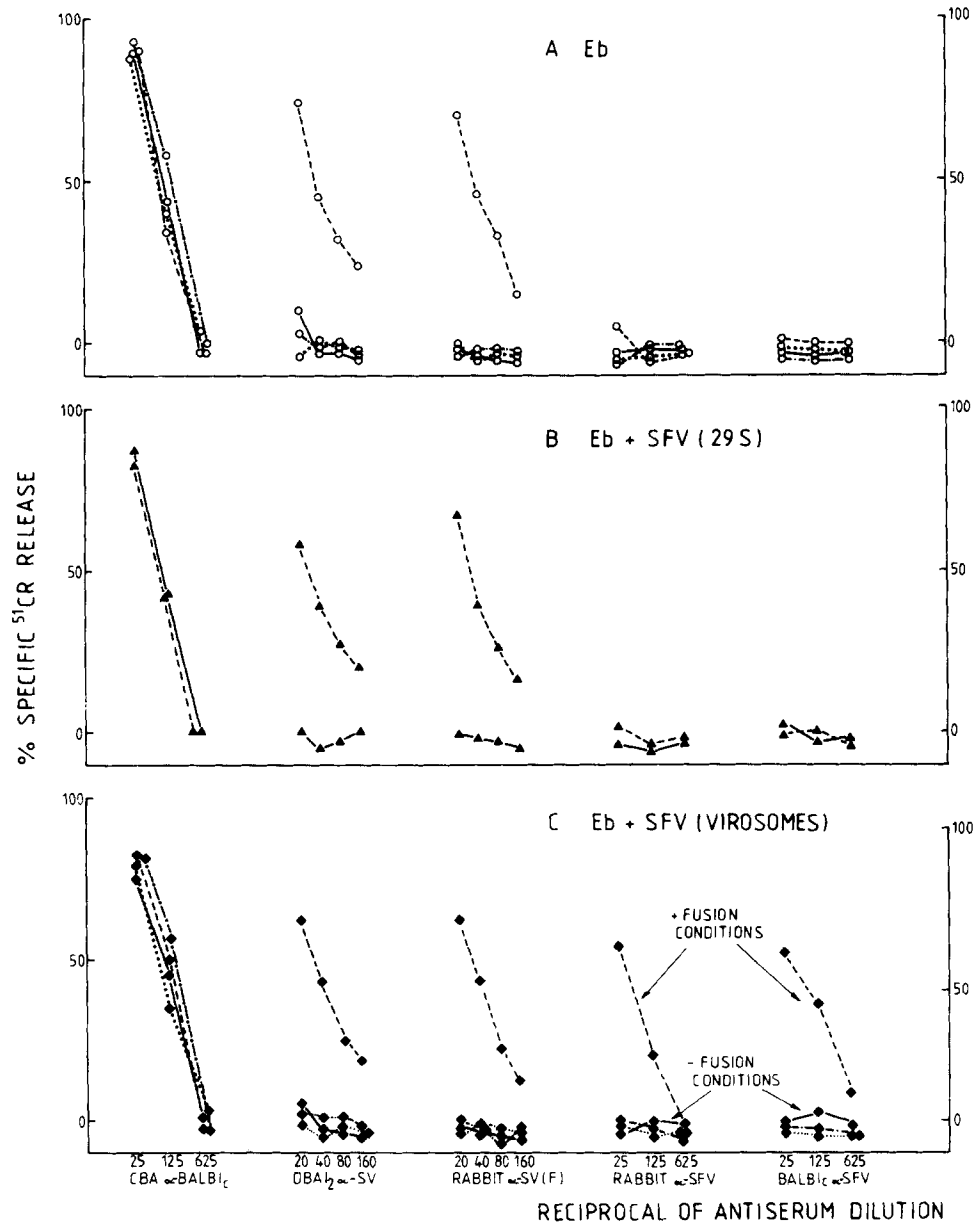


FIG. 5. CDL of Eb cells incubated with SFV-29S complexes or SFV-virosomes in the absence or presence of SV with an active or inactive F.  $25 \mu\text{g}$  of the SV preparations were added to  $10^6$  cells. (A)  $\circ$ — $\circ$ , Nontreated Eb cells;  $\circ$ — $\circ$ , Eb cells incubated with SV;  $\circ$ ... $\circ$ , Eb cells incubated with Tryp-SV;  $\circ$ — $\circ$ , Eb cells incubated with Staph-SV. (B)  $\blacktriangle$ — $\blacktriangle$ , Eb cells incubated with SFV-29S complexes;  $\blacktriangle$ — $\blacktriangle$ , Eb cells incubated with SFV-29S complexes and with SV. (C)  $\blacklozenge$ — $\blacklozenge$ , Eb cells incubated with SFV-virosomes;  $\blacklozenge$ — $\blacklozenge$ , Eb cells incubated with SFV-virosomes and with SV;  $\blacklozenge$ ... $\blacklozenge$ , Eb cells incubated with SFV-virosomes and with Tryp-SV;  $\blacklozenge$ — $\blacklozenge$ , Eb cells incubated with SFV-virosomes and with Staph-SV.

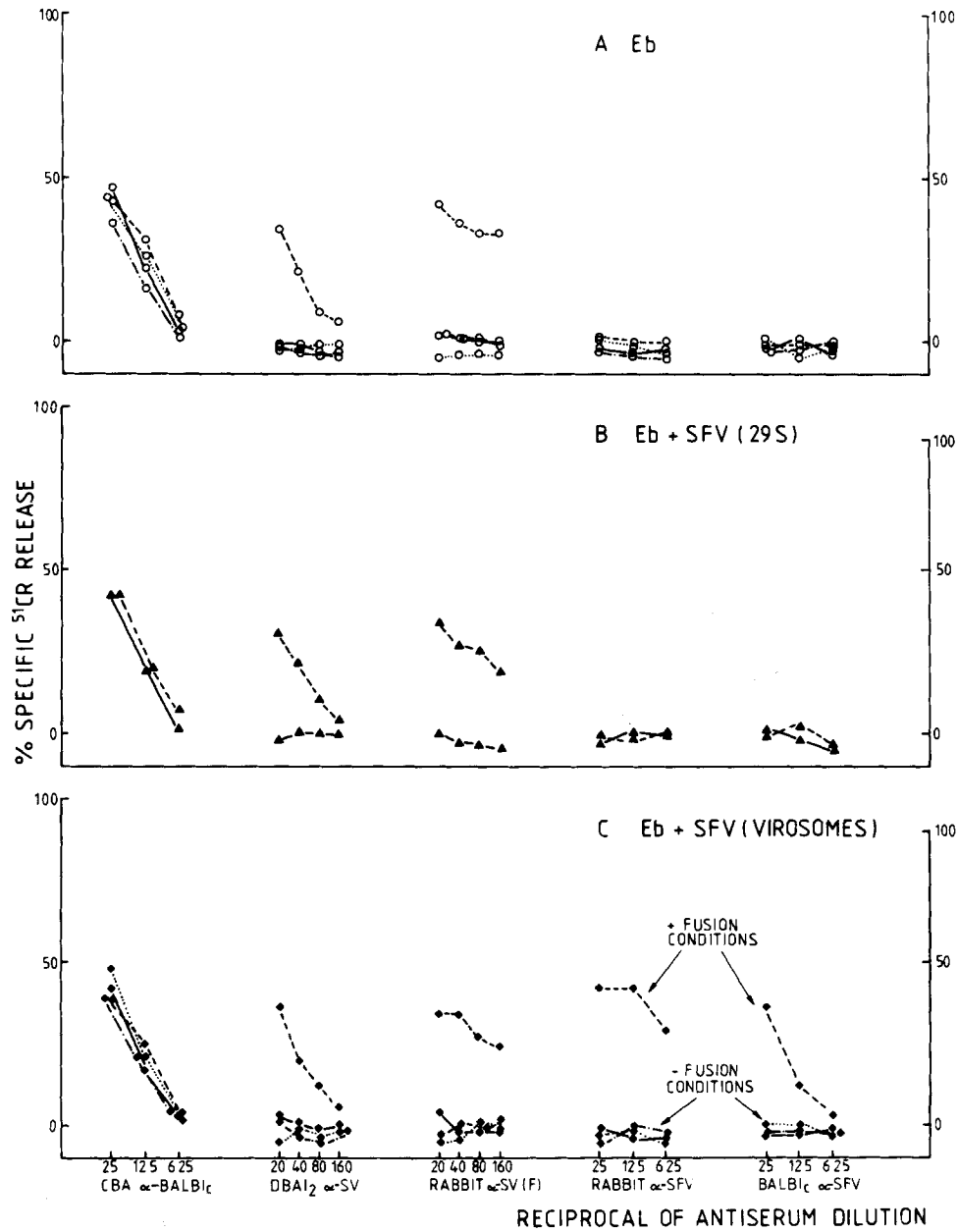


FIG. 6. ADCC of Eb cells incubated with SFV-purified spike protein as 29S complexes or virosomes in the absence or presence of SV with an active or inactive F. 25  $\mu$ g of the SV preparations were added to  $10^6$  cells. (A)  $\circ$ — $\circ$ , Nontreated Eb cells;  $\circ$ -- $\circ$ , Eb cells incubated with SV;  $\circ$ ... $\circ$ , Eb cells incubated with Tryp-SV;  $\circ$ -- $\circ$ , Eb cells incubated with Staph-SV. (B)  $\blacktriangle$ — $\blacktriangle$ , Eb cells incubated with SFV-29S complexes;  $\blacktriangle$ -- $\blacktriangle$ , Eb cells incubated with SFV-29S complexes and with SV. (C)  $\blacklozenge$ — $\blacklozenge$ , Eb cells incubated with SFV-virosomes;  $\blacklozenge$ -- $\blacklozenge$ , Eb cells incubated with SFV-virosomes and with SV;  $\blacklozenge$ ... $\blacklozenge$ , Eb cells incubated with SFV-virosomes and with Tryp-SV;  $\blacklozenge$ -- $\blacklozenge$ , Eb cells incubated with SFV-virosomes and with Staph-SV.

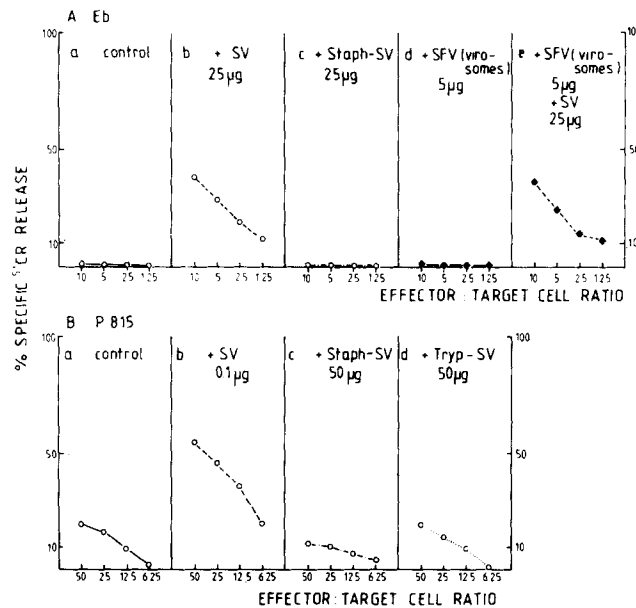


Fig. 7. SV-specific T-cell-mediated lysis of target cells incubated with SV with an active or inactive F in the absence or presence of virosomes from SFV. CTL were derived from DBA/2 spleen cells sensitized against SV. (A)  $\circ$ — $\circ$ , Nontreated P815 cells;  $\circ$ — $\circ$ , P815 cells incubated with SV;  $\blacklozenge$ — $\blacklozenge$ , P815 cells incubated with SFV-virosomes;  $\blacklozenge$ — $\blacklozenge$ , P815 cells incubated with SFV-virosomes and with SV. (B)  $\circ$ — $\circ$ , Nontreated Eb cells;  $\circ$ — $\circ$ , Eb cells incubated with SV ( $0.1 \mu\text{g}/10^6$  cells);  $\circ$ — $\circ$ , Eb cells incubated with Tryp-SV ( $50 \mu\text{g}/10^6$  cells);  $\circ$ — $\circ$ , Eb cells incubated with Staph-SV ( $50 \mu\text{g}/10^6$  cells).

cells incubated with SFV-virosomes could be lysed by anti-SFV antibodies only when SV preparations were added which could bind to and fuse with the target cell membrane. SV preparations which could still bind but not fuse because the F had been inactivated by pretreatment with trypsin did not facilitate SFV-specific lysis, even when offered in a 500-fold excess. Separate controls with anti-SV antibodies or anti-SV CTL demonstrated that only those preparations which allowed SV-specific lysis and thus contained fusion activity also facilitated SFV-specific lysis.

The stringent dependency of SFV-specific lysis on fusion between SFV-virosomes and the target cells excludes several alternative mechanisms of antigen transfer which could hitherto not be excluded in published studies on liposome cell interactions: (a) simple diffusion, which can account for transfer of lipid molecules from bilayer vesicles upon contact with cell membranes (24, 25), (b) phagocytosis, driven by metabolic energy, and (c) engulfment, driven by surface-tension effects. The data are more adequately explained by vesicle-cell membrane fusion. Although the sequence of events at the molecular level is not known, it could be anticipated to proceed via vesicle-cell attachment, lipid bilayer perturbation brought about by the F of SV, and integration of the SFV spike protein together with adjacent artificial lipid into the target cell membrane (26). Fusion activities have been reported for liposomes in the absence of a specific fusing reagent. Such liposomes had to have a low transition temperature (high fluidity) and a positive charge. However, neutral liposomes with high fluidity such as phosphatidylcholine from egg yolk as were used in the present study were reported not to fuse spontaneously with cells (27).

Recent studies with inactivated SFV rather than virosomes indicate that very similar results can be obtained (D. Barz. Unpublished observations.): target cells coincubated with SFV in the absence of a fusing reagent were resistant to SFV-specific lysis, whereas in the presence of a fusing reagent, they became susceptible. It thus seems that even the virus itself does not fuse spontaneously with the target cell membrane. It was recently demonstrated that SFV can enter target cells via endocytosis of membrane-bound viruses in coated vesicles and that these were the first steps on a pathway leading to productive cell infection (28). Together with our observations these studies indicate that SFV can enter target cells without fusing with the plasma membrane and thus without introducing susceptibility to immune cytolysis. With the help of a fusing reagent, however, it seems to be possible to induce fusion of SFV or virosomes with target cell membranes and to integrate the viral spike proteins. Electron-micrograph studies are underway to visualize this process of induced fusion.

The above interpretation of vesicle-membrane fusion could also explain why the cells coated with the SFV-29S complexes, even in the presence of a fusing reagent, could not be lysed by anti-SFV antibodies. These protein micelles, as a result of a lack of lipid cannot fuse with membranes. They are held together by the hydrophobic portions of the individual membrane proteins which interact with each other in the center of the complex. Studies by Helenius and Bonsdorff (4) and Helenius et al. (5) have shown that the 29S complexes are very stable, difficult to break, and not suitable for reconstitution into liposomes (A. Helenius. Personal communication.). Nevertheless, it could be argued that the resistance to immune lysis of the cells carrying SFV antigens merely bound to the surface is a peculiarity of the SFV system and perhaps a result of the fact that this virus binds to histocompatibility antigens. This seems unlikely because resistance to lysis was also observed with SFV-29S complexes bound to cells at low pH (6.0–6.5). At low pH, many more 29S complexes can be bound per cell than at neutral pH which was explained by additional non-specific (i.e., not H-2 mediated) binding (19). Because many of the experiments presented were performed under low pH conditions, the resistance to immune cytolysis of these cells seems to be more of general significance. Furthermore, similar results were obtained in other virus systems, such as SV used as a control in these studies and vesicular stomatitis virus (29). Lysis obtained by antisera against other antigens than the viral proteins, e.g., non-H-2 antigens, excluded the possibility of intrinsic changes in the target cell membranes or in the membrane repair system (30).

A separate finding was made when using anti-H-2 sera for control. Here a partial reduction was observed in the specific lysis when comparing 29S complex-coated with noncoated target cells. This blocking effect was (a) selective for anti-H-2 sera (directed against the private specificities of the K- and D-end molecules) and (b) more prominent with P815 than with Eb tumor target cells. The selective blocking observed with anti-H-2 sera is explained by our previous demonstration that SFV binds to H-2K and D molecules (6). The differences between Eb and P815 cells could be explained by the different numbers of SFV receptors determined ( $1.9 \times 10^4$ /Eb cell and  $0.2 \times 10^4$ /P815 cell) (19). The chance of anti-H-2 antibodies to find free H-2 determinants and to efficiently activate complement may thus be greater with Eb than with P815 cells.

The following conclusions can be reached from these studies: (a) integration of viral antigens into the lipid bilayer of target cells appears as a prerequisite for immune cytolysis; (b) immune cytolysis by antibody and complement (CDL), antibody and K cells (ADCC), and by specific CTL share this prerequisite which may indicate a

common mechanism of at least part of the lytic process; (c) cytolysis assays may serve as sensitive tests for membrane fusion and for integration of a protein into the lipid bilayer; these tests have already been used for this purpose (22, 23) but no formal proof for the validity of this assumption has existed so far; and (d) the transfer of membrane antigens via vesicles and a fusing reagent into the membranes of other cells may find a useful application in virus or tumor systems to define the target molecules involved in immune cytolysis.

### Summary

Structural requirements for membrane antigens on target cells to mediate immune cytolysis were studied in a model system with purified membrane proteins from Semliki Forest virus (SFV). These SFV spike proteins were isolated in the form of detergent- and lipid-free protein micelles (29S complexes) or, after reconstitution into lipid vesicles, in the form of virosomes. Both the 29S complexes and the virosomes were found to bind well to murine tumor cells (P815 or Eb). When these cells, however, were used as target cells in complement-dependent lysis or in antibody-dependent cell-mediated cytotoxicity assays in the presence of anti-SFV serum, they were not lysed, although they effectively bound the antibody and consumed complement. The same tumor cells infected with SFV served as positive controls in both assays.

Different results were obtained when inactivated Sendai virus was added as a fusion reagent to the cells coated with either virosomes or 29S complexes. Under these conditions the virosome-coated cells became susceptible to SFV-specific lysis, whereas the 29S complex-coated cells remained resistant. Evidence that the susceptibility to lysis of virosome-coated cells was dependent on active fusion and, therefore, integration of the viral antigens into the lipid bilayer of the target cells was derived from control experiments with enzyme-treated Sendai virus preparations.

The 29S complexes and the virosomes partially and selectively blocked the target cell lysis by anti-H-2 sera but not by anti-non-H-2 sera confirming our previous finding that major histocompatibility antigens serve as receptors for SFV. The general significance of these findings for mechanisms of immune cytolysis is discussed.

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