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Phenotypic Mixing of Envelope Proteins of the Parainfluenza Virus SV5 and Vesicular Stomatitis Virus

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Cells mixedly infected with parainfluenza virus SV5 and vesicular stomatitis virus (VSV) yield phenotypically mixed virions, in addition to both parental types. Two types of phenotypically mixed virions have been identified: 0.6 to 1.2% of the VSV plaque formers were neutralized by SV5 antiserum, but *not* by VSV antiserum, suggesting the presence of a VSV genome in an SV5 envelope; 9 to 45% of the VSV plaque formers were neutralized by *both* antisera, indicating the presence of both SV5 and VSV antigens in their envelopes. The presence of SV5 antigen in virions with the typical bullet-shaped appearance of VSV was confirmed with ferritin-labeled anti-SV5 antibody. In contrast to standard VSV, phenotypically mixed virions adsorbed to and eluted from chicken erythrocytes, indicating that these virions contained in their envelopes SV5 hemagglutinin, and possibly neuraminidase. Thus, the VSV nucleocapsid can interact with membranes which contain SV5 proteins in the manner which leads to virus maturation, and the production of a high yield of phenotypically mixed virions with the morphology of VSV indicates that this process can function efficiently. No evidence of genetic recombination between the two viruses was found. These results raise the possibility of an evolutionary relatedness between the paramyxoviruses and the rhabdoviruses.

The occurrence of phenotypic mixing between related strains of enveloped animal viruses has been demonstrated with myxoviruses (13, 14, 17), avian tumor viruses (23), and arboviruses (4). In general, phenotypic mixing has occurred between viruses of the same group, although Granoff and Hirst (15) found it with Newcastle disease and influenza viruses, which are now classified in different subgroups. Burge and Pfefferkorn (4) found no evidence of phenotypic mixing between two unrelated enveloped viruses, vesicular stomatitis virus (VSV) and Sindbis virus.

This report described the occurrence of phenotypic mixing between VSV and the parainfluenza virus SV5, two enveloped viruses that have helical nucleocapsids, but are morphologically distinct and have been classified in different groups. The presence of VSV genomes within envelopes which contain SV5 proteins, or both VSV and SV5 proteins, has interesting implications regarding virus assembly and the possible relatedness of these two viruses.

MATERIALS AND METHODS

Cells. Monolayer cultures of the MDBK line of bovine kidney cells and L929 mouse fibroblasts were grown in reinforced Eagle's medium (REM) (1) with 10% fetal calf serum, and baby hamster kidney (BHK-21-F) cells were grown in REM with 10% calf serum and 10% tryptose phosphate broth as previously described (7, 11, 18).

Viruses. The W3 strain of SV5 and the Indiana strain of VSV were grown in MDBK cells. The procedures used in inoculation of cells and harvest of total virus, including released virus and cell-associated virus released by freezing and thawing three times have been described previously (6, 7). SV5 plaque assays were done as previously described (6), except that BHK21-F cells were used with an overlay containing REM, 0.95% agar (Difco), 4% calf serum, and 2% tryptose phosphate broth. VSV plaque assays were done in BHK21-F cells as above, or in L929 cells with an overlay containing REM, 0.95% agar, and 5% fetal calf serum.

Immune sera. SV5 antiserum was prepared in rabbits as previously described (6), and rabbit anti-VSV serum was kindly supplied by Donald H. Harter of Columbia University. Sera were heated at 56 C for 30 min prior to use. Conjugation of the SV5 antiserum with ferritin was kindly performed by K. C. Hsu of Columbia University.

Neutralization tests. Virus suspensions were serially diluted in 10-fold steps in Eagle's medium with 1% bovine serum albumin, and 1 ml of each dilution was

mixed with 1 ml of a 1:100 dilution of the appropriate antisera or normal serum. Mixtures were held at room temperature for 15 min and then inoculated onto monolayers. After a 2-hr adsorption period, the inoculum was removed and the overlay was added as in the usual plaque assay procedure.

Neuraminidase. Neuraminidase from *Vibrio cholerae* was obtained from Behringwerke, Marburg, Germany.

Electron microscopy. Pellets of chicken red blood cells (RBC) with adsorbed virions were fixed in 1% glutaraldehyde in phosphate-buffered saline (PBS; reference 10) for 4 min, postfixed in 1% osmium tetroxide in PBS for 30 min, dehydrated in ethyl alcohol, and embedded in epoxy resin. Thin sections were stained with lead citrate and uranyl acetate as previously described (9).

To examine the interaction of ferritin-conjugated antibody with sectioned virions, infected cells in 30-mm plastic petri dishes were washed three times with PBS, and 0.15 ml of ferritin-conjugated SV5 antibody solution, 28 mg of protein/ml, was added to each plate. The petri dishes were held at 20 C for 40 min, with frequent tilting, and unattached material was removed by washing 10 times with PBS. The cells were then fixed with 1% glutaraldehyde for 5 min, scraped from the surfaces, and pelleted at 1,000 × *g* for 5 min. The pellets were postfixed, embedded, sectioned, and stained as above. The interaction of virions with ferritin-conjugated antibody was examined by negative staining as follows. Virus was partially purified and concentrated by sedimentation of debris at 8,000 rev/min for 20 min, followed by pelleting of virus at 20,000 rev/min for 100 min in a Spinco SW25.1 rotor. A drop of concentrated virus was mixed with a drop of the ferritin-conjugated SV5 antibody solution and incubated for 30 min at room temperature. To remove excess ferritin, the mixture was then layered over 5 ml of 5% sucrose, and the virus was pelleted by centrifugation at 35,000 rev/min for 45 min in an SW39 rotor. The pellets were rinsed gently with water and suspended in 5 ml of Eagle's medium. A drop of this suspension was placed on a Formvar-coated grid, excess liquid was removed, and a drop of 2% sodium phosphotungstate (*p*H 6.2) was applied for negative staining. All specimens were examined in a Philips EM 300 electron microscope.

RESULTS

Characterization of virus yields from cells mixedly infected with SV5 and VSV. In experiments originally designed to investigate the effects of SV5 infection of cells on superinfection with VSV, MDBK cells were inoculated with SV5 at a multiplicity of 30 to 50 PFU/cell and, after ~24 hr, were superinfected with VSV at a multiplicity of ~30 PFU/cell. After an additional 18 to 24 hr at 37 C, virus was harvested and plaque assays were performed with and without treatment with VSV and SV5 antisera. As has been previously found in rhesus monkey kidney cells (6), preinfection of MDBK cells with SV5 did not interfere with the replication of superinfecting VSV (Table 1). In the course of these experiments, it became obvious that some VSV plaque formers were being neutralized by SV5 antisera, and this phenomenon was then investigated in detail.

Table 1 shows the results of a typical experiment in which VSV plaques were quantitated after treatment with normal, anti-VSV, and/or anti-SV5 antisera. It should be emphasized that there was no risk of mistaking an SV5 plaque for a VSV plaque in these titrations, even in the absence of SV5 antiserum, since VSV plaques are larger, distinctive in appearance, appear 1 to 2 days before SV5 plaques, and are usually present at 1- to 2-log higher dilutions. Furthermore, similar results were obtained when the assays were done on L929 cells, on which SV5 will not plaque.

The top section of Table 1 shows the neutralizing capacity of the VSV antiserum; it reduced the titer of virus obtained from cells infected with VSV alone by almost 5 logs, whereas the SV5 antiserum had no effect. The bottom of Table 1 shows that the VSV antiserum failed to neutralize some of the VSV plaque-formers (compare steps B and E). On the other hand, these plaques were

TABLE 1. *Evidence for phenotypic mixing of vesicular stomatitis virus and the parainfluenza virus SV5*

Step	Yield from cells infected with	Serum used to treat virus before plaque assay	VSV plaques per monolayer[a] at dilutions of			
			$10^{-4.3}$	$10^{-5.3}$	$10^{-6.3}$	$30^{-7.3}$
A	VSV	Normal	C	C	C	74
B		Anti-VSV	2	0	0	0
C		Anti-SV5	C	C	C	73
D	VSV and SV5	Normal		C	C	71
E		Anti-VSV		48	0	0
F		Anti-VSV + anti-SV5		0	0	0
G		Anti-SV5		C	C	54

[a] Mean of plaque counts on three monolayers; C, confluent plaques.

all neutralized by the addition of SV5 antiserum (step F). The neutralization of these VSV-plaque-forming virions by SV5 antiserum, but not by VSV antiserum, suggests that they represent VSV genomes in SV5 envelopes. In this experiment there were 1.9×10^7 PFU/ml of such virions, and they represented 0.6% of the total yield of VSV plaque formers. In five such experiments, the range was 0.6 to 1.2%.

Step G of Table 1 shows that there was neutralization of VSV plaques by SV5 antiserum alone; 24% of the VSV plaques were neutralized in this experiment. Since, as shown in step E, these virions would also have been neutralized by VSV antiserum, they were doubly neutralizable virions, i.e., they had both VSV and SV5 antigens in their envelopes. Table 2 shows that the neutralization of VSV plaques by SV5 antiserum was reproducible in 10 separate experiments and was significant at the level of $P = <0.001$ (Table 2). In these experiments, 9 to 45% of the VSV plaque formers in yields from mixedly infected cells were neutralized by SV5 antiserum; the average was 27%. These results clearly show that mixedly infected cells can produce a high yield of virions which contain VSV genomes and both SV5 and VSV proteins in their envelopes. The titers of such phenotypically mixed virions in the experiments shown in Table 2 ranged from 1.6×10^8 to 1.5×10^9 PFU/ml. Thus, the presence of SV5 proteins in the membrane which becomes the viral envelope does not inhibit the maturation of VSV virions.

To investigate the possibility that genetic recombination had occurred between SV5 and VSV, VSV plaques formed by virions in the yields from mixedly infected cells were picked, and the progeny virus in these plaques were treated with SV5 antiserum to determine whether they would breed true with respect to neutralization by SV5 antibody. Forty VSV plaques were picked which had formed after treatment of the virus with VSV antiserum. Since the virions which initiated these plaques were not neutralized by VSV antiserum, *all* of these virions presumably consisted of VSV genomes in SV5 envelopes. In addition, 215 VSV plaques were picked which had formed without prior treatment of the virus with VSV antiserum; of these, ~67 would be expected to be doubly neutralizable, i.e., to have antigens of both viruses in their envelopes. The progeny virus in each of these 255 selected plaques was treated with normal and with SV5-immune sera. In no case was there neutralization by SV5 antibody; thus, none of the VSV plaques bred true with respect to the presence of SV5 antigen in their envelopes, and no evidence of recombination was obtained. Although a low incidence of recombination between these two viruses cannot be excluded by these data, it is clear that results such as those shown in Tables 1 and 2 are due to phenotypic mixing, not to recombination.

TABLE 2. *Neutralization by SV5 antiserum of VSV plaque-forming virions in the yields from cells doubly infected with VSV and SV5*

Expt no.	Serum	VSV plaques[a]	Per cent neutralized[b]
1	Normal	55	20
	Anti-SV5	44	
2	Normal	71	24
	Anti-SV5	54	
3	Normal	36	36
	Anti-SV5	23	
4	Normal	59	27
	Anti-SV5	43	
5	Normal	69	23
	Anti-SV5	53	
6	Normal	31	13
	Anti-SV5	27	
7	Normal	12	33
	Anti-SV5	8	
8	Normal	83	44
	Anti-SV5	46	
9	Normal	90	9
	Anti-SV5	82	
10	Normal	47	40
	Anti-SV5	28	

[a] Mean of plaque counts of three monolayers inoculated with a $10^{-7.3}$ dilution of virus.

[b] The neutralization by SV5 antiserum is significant, by T test, $P = <0.001$.

It would also be of interest to determine whether virions containing SV5 genomes have VSV protein in their coats, but this has proved to be technically difficult to investigate. The yield of SV5 after VSV infection, in the experiment shown in Table 1, was 4.0×10^8. However, this could be assayed only in the presence of VSV antiserum, because VSV is present in higher amounts and the plaques appear earlier, and no cell line has been found in which SV5 will plaque but VSV will not. Since it has not yet been possible to detect SV5 plaques in the absence of VSV antiserum, the question of VSV antigens in the envelope of SV5 plaque-formers remains unanswered.

Adsorption of phenotypically mixed VSV virions to chicken erythrocytes. The neutralization of the phenotypically mixed virions by SV5 antibody suggests that SV5 hemagglutinin might be among the proteins in the envelope of these virions. If this were the case, then these virions should adsorb to chicken RBC. Table 3 shows that this

TABLE 3. *Adsorption and elution from chick RBC of phenotypically mixed VSV plaque-forming virions in the yields from cells doubly infected with VSV and SV5*[a]

Virions from cells infected with	VSV plaques per ml				
	Original virus	Supernatant, post-RBC pellet	Eluate	Eluate, with neuraminidase	Infective center titration of RBC
VSV	6.0×10^8	5.8×10^8	2.0×10^6	3.2×10^6	3.5×10^5
VSV + SV5	1.7×10^9	1.0×10^9	5.0×10^8	5.6×10^8	4.2×10^8

[a] A 10-ml amount of virus was mixed with 1.0 ml of 1% chick RBC and held at 0 C for 1 hr. The cells were then pelleted, washed four times with cold PBS, and resuspended in warm PBS with or without neuraminidase, 10 units/ml, and held at 37 C for 1 hr. Portions of RBC were assayed for infective centers after the fourth wash.

did occur. Very little of the standard VSV used as a control adsorbed and eluted; however, about 40% of the VSV plaque formers in the yield from the mixed infection adsorbed, and most of these eluted from the RBC. The addition of neuraminidase to the RBC did not significantly increase the amount of virus eluted. As shown in the last column of Table 3, the adsorption of the phenotypically mixed VSV virions to RBC was also demonstrated by titering the RBC as infectious centers, and by electron microscopy as described below.

Standard VSV and the yields from mixed infection were mixed with chicken RBC and allowed to adsorb as described in Table 4. After repeated washing with cold PBS, the RBC pellets were prepared for electron microscopy. Examination of thin sections of the preparations containing the virus yields from mixed infections revealed many virions adsorbed to RBC which had the bullet-shape and striated appearance typical of VSV (Fig. 1–3), whereas no such virions were found adsorbed to RBC which had been mixed with standard VSV. Table 4 shows the quantitative results of such observations. These results not only establish the adsorption of phenotypically mixed virions to RBC, but also reveal that these virions which contain SV5 protein are morphologically indistinguishable from standard VSV virions. The latter finding supports the concept that the shape of the virion is determined by the nucleocapsid.

These adsorption experiments indicate that the phenotypically mixed VSV virions did contain SV5 hemagglutinin, and their elution from RBC suggests that they also contained neuraminidase. A direct assay for neuraminidase activity in the phenotypically mixed particles was not done because of the difficulty in obtaining a complete purification of such virions; elimination of absolutely all of the neuraminidase activity associated with the SV5 virions present in the mixed yields would be necessary before such a determination would be meaningful.

TABLE 4. *Adsorption to chick RBC of phenotypically mixed VSV-like virions from mixed infection with VSV and SV5*[a]

Virus added to RBC, yield from infection with	No. of RBC profiles examined	No. of VSV-like virions observed	
		Attached to a cell	Between cells
VSV	>600	0	0
VSV + SV5	108	83	107

[a] A 10-ml amount of virus ($\sim 10^{10}$ PFU) was mixed with 1 ml of 1% chick RBC and held at 0 C for 1 hr. The cells were then pelleted and prepared for electron microscopy.

Studies with ferritin-labeled antibody. SV5 antibody conjugated with ferritin was employed to demonstrate directly the presence of SV5 antigens in the envelopes of the phenotypically mixed, bullet-shaped VSV virions. These virions, treated with ferritin-labeled SV5 antibody, were examined both in thin section and by negative staining. For thin section studies, MDBK cells were infected with SV5, followed in 24 hr by VSV and, ~8 hr after VSV infection, were fixed and prepared for electron microscopy. Control cells were infected with VSV alone. For negative staining, virus was partially purified and concentrated as described above. Virions with the typical morphology of VSV were labeled with the SV5 antibody (Fig. 4–6). The ferritin-labeled antibody was seen widely distributed over the surface of some of the virions and appeared to be localized in patches on other virions. Some regions of the cell membranes were labeled with the antibody, whereas much of the cell surface was not labeled and presumably did not contain SV5 proteins (Fig. 4). The control VSV virions, produced by cells infected with VSV alone, were not tagged by the ferritin-labeled SV5 antibody.

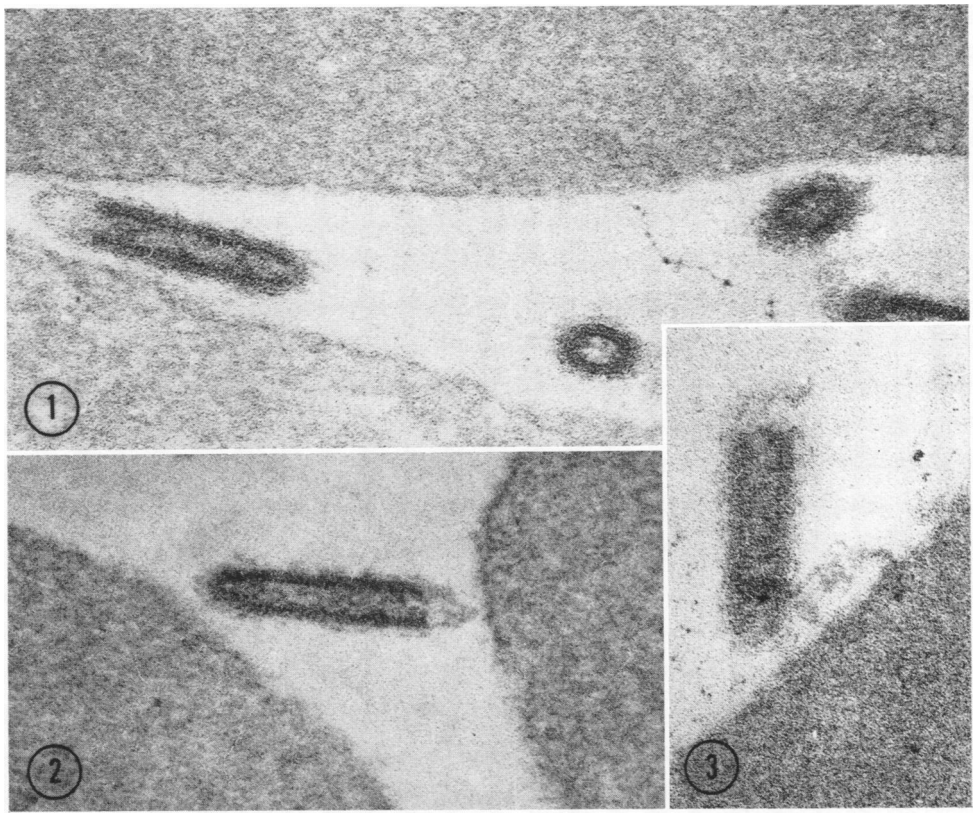

FIG. 1–3. *Adsorption to chicken erythrocytes of phenotypically mixed virions from cells mixedly infected with SV5 and VSV. The virions which have been sectioned longitudinally show the bullet shape typical of VSV. Virions appear to make contact with the cell surface on their sides or either end. The striations resulting from the coiling of the VSV nucleocapsid within the virion are evident in Fig. 3. Fig. 1 also shows some virions cut in cross section.* ×*154,000.*

DISCUSSION

The present results have demonstrated clearly that phenotypic mixing between the envelope proteins of SV5 and VSV does occur, and that hemagglutinin is one SV5 protein that can be present in virions which contain the VSV genome. The elution experiments suggest that SV5 neuraminidase may also be present in such virions, but this evidence is not conclusive. It is possible that the binding to erythrocyte receptors of phenotypically mixed virions which contain small amounts of SV5 hemagglutinin might be so weak that such particles could elute in the absence of enzymatic activity when the temperature is raised from 0 to 37 C. Such an interaction has been observed between the Lee strain of influenza virus and cat erythrocytes (22). However, it seems unlikely that all of the phenotypically mixed virions would elute in this manner, and, since treatment with neuraminidase did not increase the amount of eluted VSV plaque formers, it follows that some of the virions probably contained neuraminidase. Furthermore, 0.6 to 1.2% of the VSV plaque formers were not neutralized by VSV antiserum and, thus, presumably did not contain significant amounts of VSV envelope proteins, at least not the protein(s) with which neutralizing antibody reacts. It is probable that such virions would contain some SV5 protein in addition to hemagglutinin, since it seems unlikely that the envelope would be composed of hemagglutinin alone.

The large amounts of phenotypically mixed virions produced, particularly those with proteins of both viruses in their envelopes, indicate that the assembly of a VSV virion can proceed efficiently with SV5 proteins in the membrane which forms the viral envelope. This finding takes on added significance when considered in the light of the evidence which suggests that, under the

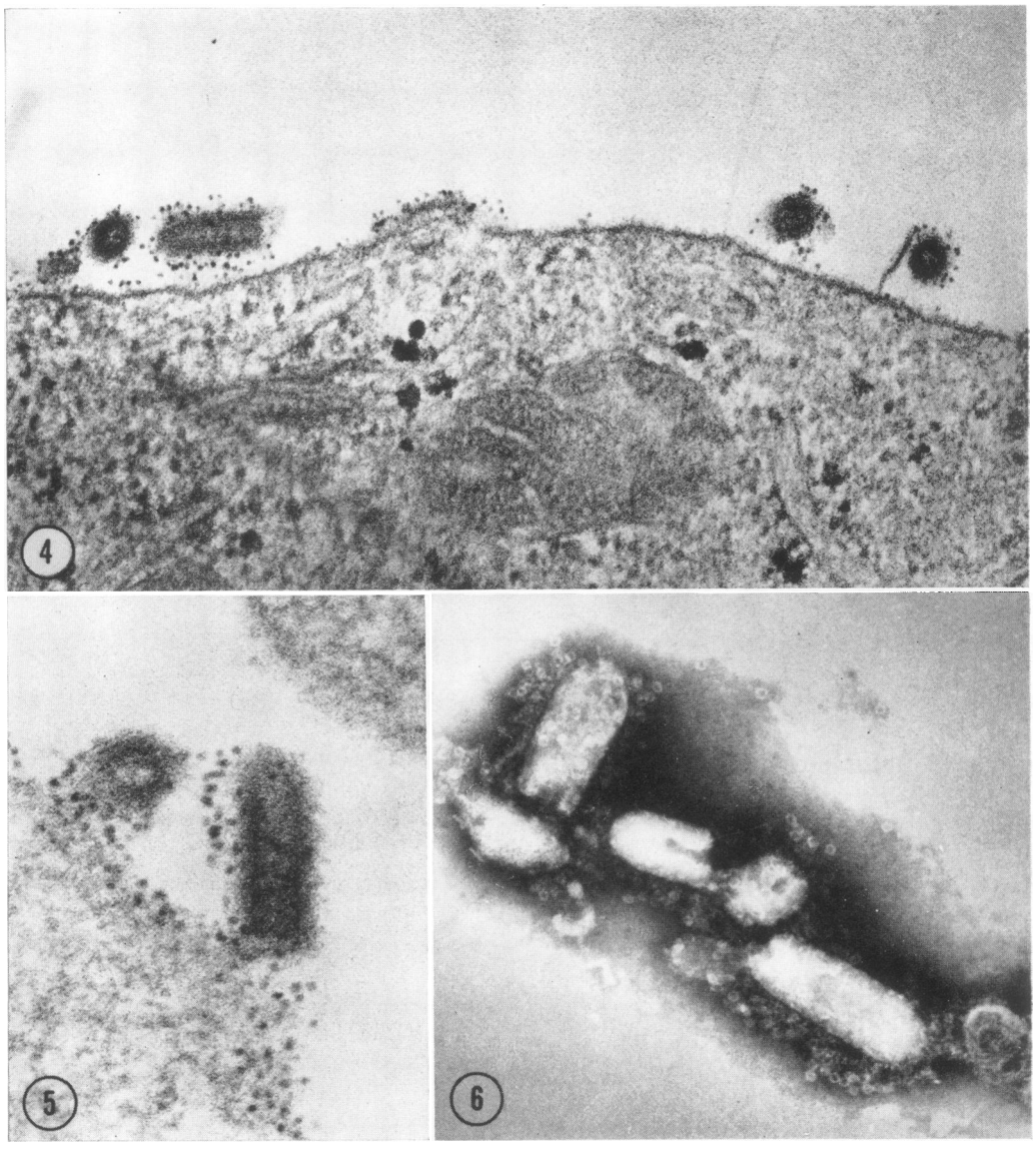

FIG. 4–6. *Phenotypically mixed virions with the morphology of VSV tagged with ferritin-labeled anti-SV5 antibody. Fig. 4. Virions with typical VSV morphology at the surface of a mixedly infected cell. The bullet-shaped virion on the left is tagged with ferritin over most of its surface, and the circular cross-sections are also clearly tagged. The cell membrane is labeled in some areas, indicating that SV5 proteins have been incorporated into the membrane. ×84,000. Fig. 5. Higher magnification of a bullet-shaped virion. The ferritin tagging is localized on one side only. ×160,000. Fig. 6. Bullet-shaped virions negatively stained with phosphotungstate. The virions are heavily labeled with the ferritin, which appears as small circles in negative contrast. ×130,000.*

usual conditions of single infection, neither of these two viruses which bud from the cell membrane contain host cell proteins in their envelopes, only virus-coded proteins (5, 24, 25). The apparent compatibility of the proteins of these two viruses within a viral envelope raises the question of some relatedness between the two viruses. This is reinforced by the fact that the current view of the maturation of the enveloped viruses is that viral nucleocapsid interacts with an area of cell membrane which contains viral proteins, and assembly by budding follows. The finding that VSV

genomes can be enclosed in envelopes which apparently contain only, or predominantly, SV5 proteins, as well as those which contain proteins of both viruses, suggests that the VSV nucleocapsid can recognize and interact in a manner which leads to virus maturation with regions of cell membrane which contain SV5 proteins. Furthermore, the high yields obtained suggest that this process functions rather efficiently. That the phenotypic mixing of the proteins of enveloped viruses shows some selectivity is suggested by the apparent lack of phenotypic mixing between VSV and Sindbis virus (4).

There are obvious structural differences between the paramyxoviruses and the rhabdoviruses; however, there are also similarities. They are both enveloped viruses with helical nucleocapsids. Though they differ in the manner in which they are coiled within the virions, the nucleocapsids of paramyxoviruses and rhabdoviruses show some similarities in structure (21), and recent evidence suggests that the protein subunits of the nucleocapsids of these viruses have similar molecular weights (2, 12, 24, 25; Mountcastle, Compans, and Choppin, *unpublished data*). VSV and SV5 both induce the production of defective, interfering particles on serial passage at high multiplicity (3, 16, 19, 20; Choppin, *unpublished data*), and the defective SV5 particles interfere with VSV as well as SV5. Finally, the base compositions of SV5 (8) and VSV (3) ribonucleic acids are almost identical; both have relatively high contents of adenine and uracil, and in both the content of adenine is similar to uracil and guanine is similar to cytosine, although the ribonucleic acids are single-stranded. In the light of the above similarities, the present results showing phenotypic mixing between SV5 and VSV are suggestive that paramyxoviruses and rhabdoviruses might be evolutionarily related.

Although the present results indicate that both SV5 and VSV proteins can be incorporated into the same regions of the plasma membrane of the host cell, it is clear that the proteins of either virus are not found throughout the entire membrane. That regions of the infected cell membrane are free of SV5 proteins was shown by the findings that large areas of the cell surface were not tagged with ferritin-labeled antiviral antibodies, and large amounts of both SV5 and VSV virions were produced which had only their own proteins in their envelopes. Similar results were found previously (6) with SV5-infected cells which were superinfected with influenza virus, another enveloped virus. These results indicate that, with enveloped viruses which bud from the cell membrane, including viruses such as SV5 which may cause persistent infection with the production of infective virus for many days (6), viral proteins appear to be incorporated into discrete, localized portions of the plasma membrane, rather than throughout the entire membrane.

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