Unilateral Phenotypic Mixing of Envelope Antigens between Togaviruses and Vesicular Stomatitis Virus or Avian RNA Tumour Virus

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SUMMARY

Vesicular stomatitis virus (VSV) in mixed infection with Sindbis virus (SbV) produces a proportion of phenotypically mixed particles (pseudotypes) containing VSV genomes and neutralization antigen(s) provided by SbV. This was demonstrated by heat-stabilization of the thermolabile t/B17 mutant of VSV and by neutralization with corresponding antisera. Phenotypic mixing is apparently unilateral because no SbV(VSV) pseudotypes could be found. Similarly, avian RNA tumour virus (ATV) in mixed infection with Sindbis virus produces a proportion of phenotypically mixed particles containing ATV genomes and SbV antigens, but no detectable particles containing SbV genomes and ATV envelope antigens. SbV acts as a helper virus for envelope-defective Rous sarcoma virus (RSV). When an avian helper virus is also present in the mixed infection, more than 90% of the RSV particles bearing SbV envelope antigens also bear ATV envelope antigens and are doubly neutralizable by antisera specific to either parent virus. In mixed infection of Langat virus and VSV, a proportion of doubly neutralizable particles containing VSV genomes were produced, but no pure pseudotypes. These results indicate that in mixed infections between enveloped animal viruses, VSV and ATV readily assemble foreign envelope glycoproteins, but that SbV does not. In certain phenotypically mixed virus stocks, only doubly neutralizable particles are found and are presumed to bear a mosaic of envelope antigens; in other stocks, particles can also be detected which are resistant to neutralization by antiserum specific to the envelope antigen encoded by their genomes, and these are presumed to represent pure pseudotypes.

INTRODUCTION

Vesicular stomatitis virus (VSV) in mixed infections with other enveloped viruses readily acquires their surface antigens. This has been demonstrated for paramyxoviruses (Choppin & Compans, 1970), myxoviruses (Závada & Rosenbergova, 1972), oncoviruses (Závada, 1972a; Huang et al. 1973; Krontiris, Soeiro & Fields, 1973; Love & Weiss, 1974) and even for herpesviruses (Huang et al. 1974); for more references see a review by Závada (1977).

The purpose of the present study was to examine the possibility of phenotypic mixing between togaviruses and other enveloped viruses. Two togaviruses were studied: Sindbis virus (SbV), belonging to the alphavirus group, and Langat virus, belonging to the flavivirus group. Burge & Pfefferkorn (1966a) failed to detect phenotypic mixing of VSV with SbV,
using a particular double mutant of SbV which phenotypically mixed with other alphaviruses. However, it is probable that only the envelope glycoproteins participate in phenotypic mixing between two enveloped viruses belonging to different groups (McSharry, Compans & Choppin, 1971; Závada & Závodská, 1973/74) and mixing of envelope glycoproteins may not have been detectable in Burge and Pfefferkorn’s experiments. In this paper we show that VSV in mixed infection with SbV does produce a proportion of virions containing the VSV genome and bearing surface antigens provided by Sindbis virus. Likewise, mixed infection of avian RNA tumour virus (ATV) with SbV produces virions containing the Rous sarcoma virus (RSV) genome and bearing SbV envelope antigens. However, no virions containing SbV genomes and VSV or ATV envelope antigens are produced.

METHODS

Viruses. VSV type Indiana, mutant tIB17 was used as previously reported (Závada, 1972b). Two stocks of SbV were used: wild-type SbV, C183 strain, was obtained from the Virus Laboratory of Rockefeller University through the courtesy of Dr M. Grešikova, WHO Collaborating Centre for Arbovirus Reference and Research, Bratislava. From this stock we isolated a large-plaque clone. The SbV temperature-sensitive mutant, ts24 (Burge & Pfefferkorn, 1966b), was kindly provided by Drs M. & S. Schlesinger (Department of Microbiology, Washington University Medical School, St Louis, U.S.A.). Bryan strain Rous sarcoma virus (RSV) produced by 16Q Japanese quail cells (Murphy, 1977) was used as an envelope-defective virus, RSV(—), or as the Rous-associated virus pseudotype, RSV(RAV-I). A highly attenuated clone of Langat virus, E15 '14' (Mayer, 1974), was kindly provided by Dr V. Mayer.

Cells. Chick embryo cells (CEC) derived from Brown Leghorn or Spafas White Leghorn flocks were used for VSV and SbV infections. The Brown Leghorn cells are frequently positive for the glycoprotein chick helper factor (chf) of the endogenous virus which may be assembled on to VSV particles to produce VSV(chf) pseudotypes (Love & Weiss, 1974). However, as none of the assay cell types used here were susceptible to VSV(chf), this factor can be ignored. Plaque assays and focus assays on avian cells were carried out as described by Závada (1972b) and Weiss, Boettiger & Murphy (1977), using CEC derived from Brown Leghorns and the Reaseheath C-line. The Brown Leghorn cells are susceptible to the avian tumour virus envelope subgroup used in this study and are therefore denoted C/O cells in this report. The Reaseheath C-line cells are selectively resistant to subgroup A avian tumour viruses and are therefore denoted C/A. Murine L cells were also used as a selective plaque assay cell type for VSV in mixed infections of VSV and SbV.

Antisera and neutralization. Hyperimmune sheep anti-VSV serum and conditions of neutralization were as described previously (Závada, 1972b). Sheep anti-SbV serum and rabbit anti-tick borne encephalitis virus serum (anti-TEV serum, which also neutralizes Langat virus) were obtained from Dr Grešikova. Pig anti-Sindbis serum was raised in an animal of approximately 30 kg immunized with virus purified by sucrose density gradient centrifugation. The first immunizing dose (approximately 2 mg of virus protein) was given in two halves: one intramuscularly with Freund’s complete adjuvant, the second half subcutaneously without adjuvant. The second immunizing dose (also approximately 2 mg) was given 5 weeks later similarly, but with incomplete adjuvant. The animal was bled 7 days after the second dose. The end-point neutralizing titre (50% neutralization of 100 p.f.u.) was approximately 1:500000.

Sindbis virus, like some other togaviruses, shows a persistent fraction after treatment
with neutralizing antiserum (Dulbecco, Vogt & Strickland, 1956). In order to achieve a more complete inactivation of SbV, the neutralization was performed in two steps (Lagwinska et al. 1975; Symington, McCann & Schlesinger, 1977). For experiments involving SbV and VSV, 0.25 ml of virus stock diluted 1:10 was mixed with 0.25 ml of anti-SbV serum, diluted 1:500 and incubated for 1 h at 37 °C. To these reacting mixtures 0.1 ml of a 2 mg/ml solution of sheep IgG hyperimmune to pig IgG was added and further incubated for 1 h at 37 °C. For experiments involving SbV and RSV similar two-step neutralization methods were employed, using rabbit anti-SbV serum and goat anti-rabbit IgG. Antiserum specific to Rous-associated virus 1 (RAV-I) was prepared in Brown Leghorn chickens (Weiss, 1969).

**Mixed infections.** Cultures of primary or secondary CEC were grown to confluence in 50 ml Müller flasks or in 90 mm Nunc plastic tissue-culture Petri dishes and were infected with VSV and/or Sindbis virus at the multiplicities indicated in Results. After virus adsorption at room temperature, the cultures were rinsed three times with buffered saline and supplied with 5 ml of Basal Eagle's or Dulbecco-Eagle's medium, containing 5% of heat-inactivated calf serum or foetal calf serum. Virus was harvested after 24 h of incubation at 31 to 32 °C.

For mixed infection of RSV and SbV, 16Q cells releasing non-infectious, glycoprotein-deficient RSV(−), or 16Q cells pre-infected 14 days previously with RAV-1 and releasing infectious RSV(RAV-1) pseudotypes, were superinfected with SbV ts24 and incubated for 24 h at 33 °C before harvest. Because attenuated Langat virus stocks have a low infectivity, the virus (approx. 10^3 p.f.u./flask) was added directly to the suspension of CEC used for seeding the cultures, and after 5 days incubation of the cultures at 37 °C, these cultures and parallel uninfected cultures were inoculated with VSV as above. Control CEC developed a complete c.p.e. within 24 h after infection with VSV, whereas cultures pre-infected with Langat virus showed no c.p.e. after superinfection with VSV, but this had little effect on the yield of VSV.

Phenotypic mixing was detected by three methods: (a) by heat-stabilization of VSV mutant t/B17 (Závada, 1972b), (b) by neutralization with specific sera (Závada, 1972a), (c) by broadening of the host range of RSV (Weiss et al. 1977), or by a combination of these methods, as described in Results.

**RESULTS**

**Selective assay conditions for VSV and SbV**

The first requirement for analysing phenotypic mixing of two viruses in a mixed infection is to find selective conditions that permit classification of the virions according to (a) the genomes they contain, and (b) their envelopes.

The classification of virions according to their genomes was based on two factors — temperature of incubation and species of cells used for assay (Table 1). In the present experiments the VSV mutant t/B17 was used throughout. This mutant has multiple temperature-sensitive defects located in genes I and V (Závada & Závodská, 1973/74). It does not revert to the wild type, and consequently produces plaques at 31 °C but not at 39 °C. The SbV used was the wild type, which plaques in chicken cells both at 31 °C and 39 °C. Thus a selective assay of particles with SbV genomes was performed at 39 °C in chicken cells. The selective assay of VSV was based on the fact that VSV produces plaques in many different species of cells, while SbV plaques in chicken cells, but not in mammalian cells, such as L or HeLa. Since the VSV used was a ts mutant, assays for VSV genomes were performed in L cell cultures incubated at 31 °C.
Table I. Selectivity of assay conditions for particles with VSV t/B17 and Sindbis virus genomes and surface antigens

<table>
<thead>
<tr>
<th>Virus</th>
<th>Treatment</th>
<th>Assay cells</th>
<th>Temp. Log\textsubscript{10} p.f.u./ml</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV t/B17</td>
<td>0</td>
<td>CEC</td>
<td>31 7.70</td>
<td>Selective assay for particles either with VSV t/B17 or with SbV genomes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CEC</td>
<td>39 &lt; 1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>L</td>
<td>31 8.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>L</td>
<td>39 &lt; 1.00</td>
<td></td>
</tr>
<tr>
<td>SbV</td>
<td>0</td>
<td>CEC</td>
<td>31 9.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CEC</td>
<td>39 9.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>L</td>
<td>31 &lt; 2.00*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>L</td>
<td>39 &lt; 2.00*</td>
<td></td>
</tr>
<tr>
<td>VSV t/B17</td>
<td>0</td>
<td>L</td>
<td>31 8.10</td>
<td>Specificity of antisera and of heat-inactivation at 45 °C</td>
</tr>
<tr>
<td></td>
<td>Anti-SbV</td>
<td>L</td>
<td>31 8.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-VSV</td>
<td>L</td>
<td>31 &lt; 1.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45 °C, 60 min</td>
<td>L</td>
<td>31 9.90</td>
<td></td>
</tr>
<tr>
<td>SbV</td>
<td>0</td>
<td>CEC</td>
<td>39 9.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-SbV</td>
<td>CEC</td>
<td>39 6.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-VSV</td>
<td>CEC</td>
<td>39 9.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45 °C, 60 min</td>
<td>CEC</td>
<td>39 9.33</td>
<td></td>
</tr>
</tbody>
</table>

* Sindbis virus stock diluted 10\textsuperscript{-1} produced diffuse, almost complete c.p.e. in L cells.

To classify virions according to their surface antigens two criteria were employed: heat lability and neutralization specificity (Table 1). VSV t/B17 carries a heat-labile defect located in the neutralization antigen (Závada, 1972b), and therefore particles carrying the glycoprotein corresponding to this mutant are inactivated by heating for 60 min at 45 °C. This treatment does not appreciably inactivate SbV as seen in Table I. The alternative method of classifying virions according to their neutralization antigens is by treatment with specific immune sera. It can be seen that hyperimmune anti-VSV (diluted 1:50 in reacting mixture) completely inactivated VSV t/B17, but had no effect on the infectivity of Sindbis virus; sheep anti-SbV serum specifically neutralized SbV but had little effect on VSV. However, neutralization of SbV was not complete; only about 2.5 log\textsubscript{10} units of infectivity were neutralized. A more complete neutralization of SbV occurred when the two-step procedure involving anti-IgG was employed (see Methods). It was sufficient to use only the anti-Sindbis serum to demonstrate the identity of the presumed VSV(SbV) pseudotype, but in attempts to find a small proportion of the expected SbV(VSV) pseudotypes among the excess particles carrying the SbV genome and SbV surface antigens, it was necessary to utilize the two-step neutralization procedure.

Phenotypically mixed particles with VSV genomes

VSV mutant t/B17 was grown in mixed infection with SbV, and simultaneously in single infection, as described in Methods. The multiplicity of infection with either virus was approximately 5 p.f.u./cell. Progeny virus was examined for the presence of phenotypically mixed VSV particles with Sindbis surface antigens by heating the virus for 60 min at 45 °C and/or by neutralization with immune anti-VSV serum. The result of this experiment is shown in Fig. 1. The permutations of the experiment are arranged in three groups: A, controls; B, treatments which should leave only VSV(SbV) pseudotypes because particles with envelope antigens corresponding to VSV only or to mosaics consisting of both VSV
Unilateral phenotypic mixing

Fig. 1. Demonstration of VSV(SbV) pseudotypes assayed under selective conditions for particles with VSV genomes. (a) Pure-grown VSV t/B17 (m.o.i. = 5). (b) VSV t/B17 (m.o.i. = 5) grown in mixed infection with SbV (m.o.i. = 5). Virus stocks were treated with anti-VSV serum, anti-SbV serum, or by heating at 45 °C for 60 min, alone or in combination, as indicated. Combinations of two antiserum were added simultaneously; heat inactivation was carried out prior to antiserum treatment. Plaques were titrated in L cells at 31 °C. Arrows indicate no detectable infectivity.

and SbV, would be inactivated; C, demonstration of the identity of VSV(SbV) pseudotypes (revealed in group B) by specific neutralization with anti-SbV serum.

Group A shows that the yield of particles with VSV genomes was decreased about ten-fold in cultures mixedly infected with SbV and that anti-SbV serum did not neutralize control VSV, and very little VSV from mixedly infected cultures. Group B shows that control, pure-grown VSV t/B17 loses more than 6 log₁₀ units of infectivity by heating at 45 °C, and even more than that by treatment with anti-VSV serum or by combination of heating with anti-VSV serum. In contrast to the pure-grown VSV, this virus mutant produced in mixed infection with SbV loses only approximately 2·5 log₁₀ units of infectivity of particles carrying VSV genomes when heated at 45 °C, reacted with anti-VSV serum, or when both treatments are combined. The surviving fraction in any of these three treatments was virtually the same. However, additional treatment with anti-SbV serum (group C) demonstrated that the particles with VSV genomes which are resistant to anti-VSV and heat treatment are indeed pseudotype particles as more than 3 log₁₀ units of infectivity are neutralized.

As an additional control, 10⁸ p.f.u. of pure-grown VSV were mixed with 10⁶ p.f.u. of pure-grown SbV (each in 9·5 ml) and incubated for 60 min at 37 °C. Subsequently, this mixture was heated and/or treated with anti-VSV as virus stocks used in Table 1. No plaques appeared after plating this mixture undiluted or in 10⁻¹ and 10⁻² dilutions under selective conditions for particles with VSV genomes. This showed that the VSV(SbV) pseudotype results only from intracellular interaction between the two viruses.
Fig. 2. Failure to demonstrate SbV(VSV) pseudotypes assayed under conditions selective for particles with SbV genomes. (a) Pure-grown SbV (m.o.i. = 5). (b) Product of a cell culture mixedly infected with SbV (m.o.i. = 5) and VSV t/B17 (m.o.i. = 5). (c) Product of a cell culture mixedly infected with SbV (m.o.i. = 5) and with VSV t/B17 (m.o.i. = 50). Viruses were treated with sheep anti-VSV serum, pig anti-SbV serum, or with IgG isolated from sheep serum immunized with pig IgG, alone or in combination, as indicated. Combinations of antiviral sera were added simultaneously; anti-pig IgG was added after incubation with antiviral sera. Plaques were titrated in CEF cultures at 39 °C.

Failure to detect particles with Sindbis virus genomes and VSV neutralization antigens

The results of an experiment aimed at detecting the reciprocal pseudotypes, i.e. SbV(VSV), are presented in Fig. 2. Simultaneous infection of cells with VSV reduced the total yield of particles with SbV genomes (columns of groups A), but did not markedly affect the fraction neutralized by anti-SbV serum with or without anti-pig IgG (columns of groups B). The fractions of virions with SbV genomes, either pure-grown or produced in mixed infection with VSV, that were neither neutralized with anti-SbV serum alone nor with this serum plus anti-pig IgG, did not show any further decrease of infectivity when treated with sheep anti-VSV serum (columns in groups C). These results demonstrate that if there are any SbV(VSV) pseudotypes at all, their frequency is below the level of detection, i.e. a frequency of less than $10^{-5}$ among all virions containing SbV genomes.

Phenotypic mixing of VSV with Langat virus

As a representative of the flaviviruses an attenuated variant of Langat virus was chosen for mixed infection with VSV. This variant multiplies poorly in tissue cultures and therefore cells were infected with Langat virus 5 days before superinfection with VSV. Cells pre-infected with Langat virus showed no c.p.e. after superinfection with VSV, but the titres of VSV were not much reduced in these compared to uninfected controls. In two experiments the VSV yields were 2·5 and $1·2 \times 10^8$ p.f.u./ml from singly-infected cultures, and 8·0 and $3·1 \times 10^7$ p.f.u./ml from cultures pre-infected with Langat. Either method of
### Unilateral phenotypic mixing

#### Table 2. Phenotypic mixing of VSV with Langat virus

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Virus</th>
<th>Added serum</th>
<th>Mean plaque counts</th>
<th>% Infectivity neutralized</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VSV pure-grown</td>
<td>Normal rabbit</td>
<td>373</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Anti-TEV</td>
<td>402</td>
<td></td>
<td>$-7.8 (P &gt; 0.05)$</td>
</tr>
<tr>
<td>VSV+Langat</td>
<td>Normal rabbit</td>
<td>390</td>
<td></td>
<td>--</td>
</tr>
<tr>
<td>Mixed infection</td>
<td>Anti-TEV</td>
<td>281</td>
<td></td>
<td>$27.9 (P &lt; 0.01)$</td>
</tr>
<tr>
<td>2</td>
<td>VSV pure-grown</td>
<td>Normal rabbit</td>
<td>397</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Anti-TEV</td>
<td>402</td>
<td></td>
<td>$-1.3 (P &gt; 0.05)$</td>
</tr>
<tr>
<td>VSV+Langat</td>
<td>Normal rabbit</td>
<td>465</td>
<td></td>
<td>--</td>
</tr>
<tr>
<td>Mixed infection</td>
<td>Anti-TEV</td>
<td>319</td>
<td></td>
<td>$31.4 (P &lt; 0.01)$</td>
</tr>
</tbody>
</table>

detecting presumed VSV(Langat) pseudotypes (heating at $45\,^\circ\mathrm{C}$, or neutralizing with anti-VSV serum) showed that these did not occur at titres higher than 10 p.f.u./ml.

Therefore assays were set up to test the occurrence of doubly neutralizable particles bearing the VSV genome and a mosaic surface consisting of both VSV and Langat virus antigens. Virus yields from two experiments were diluted to contain 10000 p.f.u./ml when mixed with rabbit anti-TEV serum or with normal rabbit serum (final dilutions in reaction mixtures 1:25) and incubated for 60 min at $37\,^\circ\mathrm{C}$. After this, reaction mixtures were diluted 1:10, and 0.5 ml volumes were plated for plaque-forming units in sets of 10 parallel 100 mm plates. The results (Table 2) demonstrate that whereas pure-grown VSV is not significantly neutralized with anti-TEV serum, approximately 30% of the VSV particles are neutralized with anti-TEV serum when the VSV was propagated in cultures pre-infected with Langat virus. These particles neutralized with anti-TEV serum are apparently particles with a mosaic of surface antigens, since all plaque-forming units in conditions selective for VSV genomes were neutralized with anti-VSV serum.

#### Unilateral phenotypic mixing between Sindbis virus and avian RNA tumour virus

In mixed infections between VSV and Rous sarcoma virus, reciprocal pseudotypes are formed (Weiss et al. 1977). Particles carrying VSV genomes and RSV envelope antigens, denoted VSV(RSV), can be detected as plaque-forming units resistant to anti-VSV serum or to heat treatment, while particles carrying RSV genomes and VSV envelope antigens, denoted RSV(VSV), can be detected as focus-forming units with an extended host range. Experiments were therefore set up to investigate whether Sindbis virus and avian tumour viruses will mix phenotypically.

The Bryan high titre strain of RSV is defective in envelope antigen and a helper virus is required for the production of infectious RSV pseudotypes. The transformed quail cell line 16Q releases non-infectious RSV(−) particles but, on superinfection with the subgroup A helper virus, RAV-1, produces a high titre of RSV(RAV-1) pseudotype. RSV(RAV-1) is infectious in Brown Leghorn C/O cells but is not infectious in Reaseheath C-line C/A cells because C/A cells lack the gene that determines cell surface receptors for viruses with subgroup A envelope specificity. SbV, however, will infect both C/O and C/A cells. The assembly of SbV envelope antigens on to RSV(−) particles should produce RSV infectious for C/O and C/A cells, and the assembly of SbV antigens on to RSV(RAV-1) should render those particles infectious for C/A cells. RSV(SbV) pseudotypes should therefore be detectable as an extension of host range. The reciprocal pseudotype particles with SbV genomes and avian tumour virus envelopes should be detectable as a fraction resistant...
Fig. 3 shows the results of mixed infections between RSV and SbV. 16Q cells releasing RSV(−) or RSV(RAV-I) were superinfected with the temperature-sensitive mutant, SbV\textsubscript{ts24} at a multiplicity of 3 p.f.u./cell and incubated at 33 °C for 24 h when medium was harvested for virus assays. SbV was assayed as plaque-forming units incubated at 31 °C. Approximately equivalent titres of SbV were obtained on growth in cells releasing either RSV(−) or RSV(RAV-I), and the plating efficiency was reduced about twofold in either case on C/A cells as compared to C/O cells. Treatment with rabbit anti-SbV serum reduced the titre by approximately 2 \( \log_{10} \) units of infectivity, and subsequent addition of anti-rabbit IgG reduced the infectivity by a further 3 \( \log_{10} \) units. Treatment with chick anti-RAV-I serum alone or in combination with anti-SbV serum and anti-rabbit IgG did not influence the SbV titres. There was no significant difference in antiserum-resistant titres between SbV grown in cells producing RSV(−) or RSV(RAV-I), whether assayed on C/O or C/A cells. Thus it appeared that RAV-I envelope antigens are not assembled on to SbV particles because neither pseudotypes nor doubly neutralizable plaque-forming units were detected.

Fig. 3 also shows the titres and host range of RSV following double infection with SbV. RSV was assayed in C/O and C/A cells as focus-forming units (f.f.u.) incubated at 41 °C. The high temperature (though physiological for chick cells) was used in order to prevent

- to anti-SbV serum. No SbV pseudotypes would be expected to result from mixed infection with RSV(−) because the RSV is defective in envelope antigen, but after mixed infection with RSV(RAV-I), SbV(RAV-I) might be formed; it would be expected to plate on C/O cells but not on C/A cells.

Fig. 3 also shows the titres and host range of SbV (p.f.u.) and RSV (f.f.u.) in harvests of mixed infections treated with antiserum and assayed on C/O (●) or C/A (■) chick cells as indicated. (a) Virus harvested from quail cells releasing non-infective RSV (−) and superinfected with SbV\textsubscript{ts24} (m.o.i. = 5). (b) Virus harvested from quail cells releasing RSV(RAV-I) with SbV superinfection. (c) Pure-grown RSV(RAV-I). Arrows signify no infectivity in C/O or C/A cells, and dashed arrows, no infectivity in C/A cells.

To summarize, mixed infections between RSV and SbV resulted in similar titres of SbV when harvested from cells releasing RSV(−) or RSV(RAV-I). Treatment with anti-SbV serum reduced the titre by approximately 2 \( \log_{10} \) units of infectivity, and subsequent addition of anti-rabbit IgG reduced the infectivity by a further 3 \( \log_{10} \) units. There was no significant difference in antiserum-resistant titres between SbV grown in cells producing RSV(−) or RSV(RAV-I), whether assayed on C/O or C/A cells. Thus it appeared that RAV-I envelope antigens are not assembled on to SbV particles because neither pseudotypes nor doubly neutralizable plaque-forming units were detected.
replication of the excess SbV ts24 present in the mixed harvests. After superinfection with SbV ts24, harvests of RSV(-)-releasing cultures titred approximately $10^6$ f.f.u./ml on C/O cells, whereas control RSV(-) culture fluids had no infectivity. As with SbV plaque assays, the titre on C/A cells was approximately twofold lower than on C/O cells. The f.f.u. titre on both cell types was eliminated by treatment with anti-SbV serum but was unaffected by anti-RAV-1 serum. The infectivity is attributed to the formation of RSV(SbV)pseudotypes.

Superinfection of cells releasing RSV(RAV-1) by SbV also resulted in phenotypically mixed RSV particles (Fig. 3). RSV(RAV-1) harvested without SbV infection titred more than $7 \log_{10}$ units on C/O cells but had no infectivity for C/A cells. In contrast, $3.6 \log_{10}$ units of infectivity for C/A cells appeared after SbV superinfection. This infectivity on C/A cells was completely eliminated by treatment with anti-SbV serum [which did not affect the plating of RSV(RAV-1) on C/O cells] indicating that the extension of host range was attributable to the assembly of SbV envelope antigens on to RSV particles. However, more than 90% of the f.f.u. on C/A cells was also neutralized by treatment with anti-RAV-1 serum. This result indicates that less than 10% of the phenotypically mixed RSV progeny represented pure RSV(SbV) pseudotypes, the remainder comprising RSV with mosaic envelopes bearing both SbV and RAV-1 antigens, and being subject to neutralization by either anti-SbV or anti-RAV-1 serum.

Additional experiments (not shown here) in which SbV was propagated in uninfected chick cells and in chick cells producing high titres of three other avian tumour viruses, RAV-1, RAV-49 (subgroup C) or Carr-Zilber associated virus (subgroup D) failed to reveal any evidence of the assembly of avian tumour virus antigens on to virions carrying SbV genomes. When pure-grown stocks of RSV(RAV-1) and SbV were incubated for 1 h at 37 °C before assay, no extension of RSV host-range was observed. Thus it appears that, as with mixed infections of VSV and SbV, mixed infections of RSV and SbV result in unilateral phenotypic mixing, in which only the oncovirus acquires the unrelated envelope antigen.

**DISCUSSION**

The present paper extends previous findings that VSV and RSV readily mix phenotypically with other enveloped viruses to the group of togaviruses. The difference between Sindbis virus (alphavirus) and Langat virus (flavivirus) is in the finding that VSV forms complete pseudotypes with the former, whereas with the latter, VSV, forms only doubly neutralizable particles. This could probably be explained on the basis of the difference of yields of these viruses. SbV infections produce yields of the order of $10^6$ p.f.u./ml, whereas the Langat clone used yielded only about $10^5$ p.f.u./ml. The failure of Langat to produce a complete pseudotype with VSV with which it yields doubly neutralizable particles can be explained on statistical grounds (Závada, 1977). Doubly-neutralizable particles are also found for RSV bearing VSV antigens (Weiss et al. 1977) or SbV antigens, as reported here. In these cases, only a low antiserum-resistant fraction is found despite the high titre of the donor virus of envelope antigens, but the extension of RSV host range gives a sensitive assay for both pure pseudotype and doubly neutralizable particles. Assuming single-order kinetics of the neutralization of enveloped viruses by antibody (Dulbecco et al. 1956), the binding of antibody molecules to antigens of one specificity in a mosaic envelope should effectively neutralize the particle, even if that antigen does not participate in interaction with the cell surface receptor. This would explain why the focus-forming units on C/A cells are neutralized by anti-RAV-1 serum following mixed infection of SbV and RSV(RAV-1) but not following mixed infection of SbV and RSV(-). However, in an earlier study of phenotypic
mixing between avian tumour viruses of different subgroups, Vogt (1967) interpreted a high persistent fraction after treatment with one antibody as evidence for mosaic particles. A careful analysis of the kinetics of neutralization of RSV pseudotypes may help to elucidate the properties of particles with mosaic envelopes.

There is an apparent contradiction of the present results with an earlier report (Burge & Pfefferkorn, 1966a) on the failure to detect phenotypic mixing between VSV and SbV. In the earlier experiments a double mutant of SbV, HR-ts (heat-resistance and temperature-sensitivity for growth) was employed, and it was not known which of the virus-specified proteins were altered by these mutations. Using this double mutant, it was possible to demonstrate phenotypic mixing with wild-type forms of SbV and with Western and Eastern equine encephalomyelitis viruses, all of which are members of the alphavirus group. Phenotypically mixed virions were revealed by heating at 60 °C (virions containing the HR protein of the mutant), and by plating for plaques at 39 °C (genomes of wild-type viruses). Between these closely related viruses, any virus protein might be exchanged in phenotypic mixing or complementation. But in phenotypic mixing between various groups of enveloped viruses, only surface glycoproteins are exchanged (McSharry et al. 1971; Závada & Závodská; 1973/74). Moreover, in contrast to togaviruses, VSV is a negative-strand RNA virus; it carries in its virions an RNA polymerase which is the most thermolabile component in wild-type VSV (Marcus & Sekellick, 1975), being inactivated at 50 °C. Thus, all particles carrying VSV genomes together with VSV polymerase must have been inactivated under Burge & Pfefferkorn's conditions. Our present experiments were directed toward detecting phenotypic mixing at the level of surface antigens, and this easily explains the apparent contradiction.

Another point is the failure to detect the reciprocal pseudotypes, SbV(VSV) and SbV-(RAV-I), although reciprocal pseudotypes between VSV and oncoviruses have been detected (Weiss et al. 1977; Livingston, Howard & Spence, 1976). However, pseudotypes of SbV bearing envelope antigens of lactic dehydrogenase virus, a flavivirus, have been demonstrated (Lagwinska et al. 1975). A similar polarity of pseudotype formation was previously observed in mixed infection of VSV with fowl plague virus (Závada & Rosenbergova, 1972). It is possible that in vitro mixing with virus membrane preparations (Bishop et al. 1975) will yield SbV-containing particles with the characteristics of pseudotypes, as the glycoprotein-lipid complexes may envelope the virus particles to form infectious vesicles. However, such vesicles do not appear to form in vivo under the conditions used here for mixed infections. At present, we can only surmise that the explanation for unilateral phenotypic mixing might be sought in the specificity of assembly of virus cores with envelopes. The envelope of togaviruses appears to be closely bound to the nucleocapsid; it is possible that the envelope glycoproteins associate directly with the core proteins, since there is no SbV equivalent of the VSV M-protein. Sindbis virus may therefore have a more specific requirement for the assembly of related togavirus envelope glycoproteins than have VSV and oncoviruses, which exhibit less stringent recognition. Further studies of phenotypic mixing between unrelated viruses may prove valuable in elucidating the specificity of interaction and assembly of membrane glycoproteins.

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Unilateral phenotypic mixing

REFERENCES


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