Rescue of Presumptive Viral Information from Human Cells by a Helper Oncovirus

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SUMMARY
We have attempted to rescue presumptive human endogenous retrovirus(es) by using a competent animal oncovirus as a helper. Human melanoma cells (line HMB2) were fused, using polyethylene glycol, with mouse NIH-3T3 cells which had been infected and transformed by the Harvey murine leukaemia and sarcoma virus complex (MLV and MSV). The heteropolykaryons obtained were co-cultivated with fresh NIH-3T3 cells; filtered (Millipore 0.22 μm) medium from these was used to infect further NIH-3T3 cells. In these cells after several passages, vesicular stomatitis virus (VSV) pseudotypes could be produced. These were infectious not only for mouse cells (manifesting the helper MLV), but also for human cells (HeLa, HEC human embryo fibroblasts, HMB2); they were not infectious for CCL64 (mink) or for Vero (African green monkey) cells. The presence of such VSV pseudotypes infectious for human cells indicated that a human ecotropic virus [provisionally named rescued human virus (RHV)] had been rescued by the fusion of human melanoma cells with MLV-infected mouse cells. This was supported by the following evidence. (i) The human-specific pseudotype was neutralized by sheep antisera raised to antigens selected by VSV from human tumour cell lines HMB2, T47D and HeLa. (ii) These antisera also aggregated NIH cells infected with MLV and RHV. (iii) Mouse antisera raised to antigens present in NIH cells infected with MLV and RHV, in contrast to sera raised to NIH cells infected with MLV only, immunoprecipitated an 85,000 mol. wt. protein band from human cells (HEC, HMB2 and HeLa) surface-labelled with 125I.

INTRODUCTION
There has been an intensive search for endogenous human oncoviruses, and much evidence for their existence has gradually accumulated from studies using electron microscopy, tests for reverse transcriptase, reactions for antigens cross-reactive with known animal oncoviruses, cloning of segments of human DNA that show sequence homology with animal retroviruses etc. (for reviews, see Weiss, 1984, 1985). However, to our knowledge, there has been no evidence so far of infectious, competent virions, or of defective retrovirus information rescued by a competent helper virus and transmitted to other cells, human or non-human.

Therefore, in the present work we attempted to rescue presumptive endogenous retrovirus(es) from human cells by using a competent helper oncovirus, and to transfer it into non-human cells. As a source of this human retrovirus we used a human melanoma cell line (HMB2), and Moloney mouse leukaemia virus as a helper. [In fact, we used the Harvey murine leukaemia and sarcoma virus (MLV and MSV) complex; the defective MSV component was included as a convenient indicator to show by transformation that the helper virus had multiplied sufficiently.] Since Moloney MLV is not infectious for human cells, we fused melanoma cells with mouse NIH-3T3 cells, previously infected and transformed by MLV and MSV (NIH + MLV + MSV), using polyethylene glycol (PEG 6000). Fresh NIH-3T3 cells, permissive for the helper MLV, were added to the heteropolykaryons obtained.

As a first test to detect the transfer and expression of human viral information in mouse cells, we employed the formation of vesicular stomatitis virus (VSV) pseudotypes. There were three
reasons for this choice. First, VSV(oncovirus) pseudotypes have a host range determined by the oncovirus used. Since the VSV(MLV) pseudotype is infectious only for mouse cells (Závada, 1982; Závada et al., 1977), the appearance of VSV pseudotypes infectious for cells derived from other species would reveal the presence of another enveloped virus, distinct from MLV. Second, even a defective virus can provide envelope antigens for VSV pseudotypes (Love & Weiss, 1974). In other species would reveal the presence of another enveloped virus, distinct from MLV. Second, even a defective virus can provide envelope antigens for VSV pseudotypes (Love & Weiss, 1974). Third, pseudotype neutralization could enable further characterization of the rescued envelope donor.

Indeed, the host range of VSV pseudotypes produced in NIH-3T3 cells co-cultivated with the heteropolykaryons described above indicated that an agent distinct from the helper MLV (as well as from xenotropic MLV) had been recovered. This was provisionally named rescued human virus (RHV). In association with the helper MLV, it was transmissible into further NIH-3T3 cells via filtered tissue culture medium (i.e. NIH-F4 culture).

Three points supported the view that the recovered agent was of a viral nature: its rescue by helper MLV; its capacity to form functioning, infectious VSV pseudotypes; its filtrability through Millipore 0.22 μm membranes.

To test whether the rescued agent was of human origin, we used sheep antisera previously raised to antigens assembled into the virions of VSV [or HVJ (Sendai virus or haemagglutinating virus of Japan)] from human cells (Závada et al., 1983, Závada & Huang, 1984). The rationale for this was our hypothesis (Závada, 1982) that enveloped viruses (including VSV) preferentially assemble virus-related proteins from the host cells. In turn, we produced antisera directed to antigens of RHV expressed in NIH-F4 cells, and used them in radioimmunoprecipitation of the corresponding proteins from human cells.

It is our feeling that the results obtained so far and presented in this paper do not definitively demonstrate rescue of a human endogenous virus, although none of them contradicts this possibility. Nevertheless, they describe an apparently novel agent, the biological significance and identity of which remain to be determined.

**METHODS**

**Viruses.** Standard VSV (Indiana serotype), Harvey MLV and MSV, Moloney MLV and xenotropic mouse leukaemia virus (X-MLV) were as described previously (Závada, 1972; Závada et al., 1977). The retroviruses were maintained in persistently infected cells, MLV alone and the MLV and MSV complex in NIH-3T3 cells, X-MLV in mink cells.

**Cells and media.** Cell lines NIH-3T3, Vero, mink (CCL64), human embryo cells HEC (passage levels 5 to 10), HMB2 (derived from a melanoma), HeLa cells and all the media have been described previously (Závada et al., 1977, 1983).

**VSV pseudotypes.** These were produced as before (Závada et al., 1977) by growing VSV in cells persistently infected with the appropriate oncovirus. Infectious tissue culture fluid was harvested 6 h after infection of cells with VSV, clarified and frozen in aliquots at −70 °C. Since the VSV(RHV) pseudotype was unstable at −70 °C, it was always prepared freshly for each experiment and stored only overnight at 4 °C. The titres of VSV(RHV) assayed in HeLa cells were always between 1.0 × 103 and 1.5 × 103 p.f.u./ml of infectious tissue culture fluid. VSV pseudotypes were assayed after mixing appropriate dilutions of virus stocks and anti-VSV serum; these dilutions were prepared in Dulbecco's complete phosphate-buffered saline (PBS) supplemented with 1% foetal calf serum and 30 μg/ml DEAE-dextran 6000 (Pharmacia). Before plating for plaque formation, the virus and serum mixtures were incubated at 37 °C for 60 min. As a special precaution, virus preparations for some experiments (e.g. that shown in Fig. 1) were filtered through a Millipore 0.45 μm membrane before use. This was done because we have previously found (unpublished) that such filtration removes any occasional traces of non-pseudotype VSV infectivity that is resistant to both antisera (anti-VSV and anti-oncovirus).

**Antisera.** Sheep antisera to wild-type VSV (grown in lamb cells) and to human cell proteins assembled by the VSV ts-O45 mutant at non-permissive temperature from the human tumour cell lines (anti-HMB2, anti-T47D and anti-HeLa) and to antigens of HMB2 cells assembled by HVJ [anti-(HVJ)HMB2] have been described previously (Závada et al., 1983; Závada & Huang, 1984). Sheep anti-MLV serum was produced following the same schedule; MLV for immunization was produced in NIH-3T3 cells and gradient-purified. Mouse sera against MLV and against MLV and RHV were obtained from BALB/c mice which had been immunized with suspensions of NIH-3T3 cells infected with MLV or with MLV and RHV, disintegrated by sonication (Raytheon sonic oscillator, 10 kHz 150 W) twice for 30 s. Antigens for the first immunizing dose were emulsified with complete and for the second (6 weeks later) with incomplete Freund's adjuvant. The amount of antigen used for each immunizing dose corresponded to 106 cells per mouse. The mice were bled 8 days after the second immunizing dose.
**Fusion of cells and rescue of RHV.** Formation of heteropolykaryons in a dense mixed monolayer culture of NIH + MLV + MSV and of HMB2 cells (approx. 1:1) was induced by PEG 6000 by the method of Davidson & Gerald (1976). Mixed cell culture was treated with 50% (v/v) PEG 6000 (Lachema, Czechoslovakia) for 3 min. This induced the majority of cells to fuse into large polykaryons (10 to 20 nuclei each) within 3 h of subsequent incubation. At this time fresh NIH-3T3 cells were added and the culture was further incubated for 3 days. It was subsequently passed five times. At this stage, the first evidence of VSV(RHV) pseudotypes formation was obtained, but the culture still contained some cells with a human karyotype. Therefore, medium from this culture was filtered (Millipore 0.22 μm) and after adding Polybrene (15 μg/ml) was used undiluted for infection of fresh NIH-3T3 cells. After five blind passages, these cultures again were found to give rise to the VSV(RHV) pseudotype. Since their production, these cells (designated NIH-F4) have been kept as a cell persistently infected with MLV and MSV and RHV and were used in all experiments described in Results. They exhibit a mouse karyotype only.

**Surface radioiodination of cells.** This was performed as described (Salisbury & Graham, 1981). One-hundred μCi of 125I (carrier-free, Izinta, Budapest, Hungary) was added to 0.5 ml of an approximately 20% (v/v) suspension of cells in PBS pH 7.2, in 4 cm glass Petri dishes coated with iodogen. After 20 min of iodination, the cell suspension was diluted and centrifuged (3000 r.p.m. for 3 min), and rinsed once more with PBS. The cell pellets were extracted with RIPA buffer [0.14 M-NaCl, 6.7 mM-phosphate buffer pH 7.2, 1% Triton X-100, 0.1% deoxycholate, 1 mM-phenylmethylsulphonyl fluoride, 20 μl/ml Antilysin (Spofa, Czechoslovakia; equivalent to Trasylol)].

**Immunoprecipitation and polyacrylamide gel electrophoresis (PAGE).** These were performed as described by Kessler (1975) and Laemmli (1970). For autoradiography, the gels were exposed on Medix Rapid films (Czechoslovakia) with intensifying screens at −70°C. As molecular weight markers, the Sigma kit (product no. MW-SDS-200) was used: 29K, carbonic anhydrase; 45K, ovalbumin; 66K, bovine serum albumin; 97-4K, phosphorylase b; 116K, β-galactosidase; 205K, myosin).

**RESULTS**

**Host range of the VSV(RHV) pseudotype**

VSV produced in NIH-3T3 cells, NIH-3T3 cells infected with MLV and MSV, and in NIH-F4 cells was plaque-assayed for total and for pseudotype infectivity in seven different cell cultures, as shown in Fig. 1. The pattern of total VSV infectivity for these cells was very similar for all three virus stocks. No pseudotype at all was detected in preparations of VSV produced in control NIH-3T3 cells. The infectivity of VSV pseudotypes produced in NIH + MLV + MSV cells corresponded both in its host range and interference specificities to VSV(MLV); the appearance of a few plaques in mink cells perhaps reflected a very low level of expression of gene products corresponding to those of xenotropic MLV or mink cell focus-forming virus. VSV produced in NIH-F4 cells appeared to contain two different pseudotypes simultaneously. One of these was the same as in the previous stock, and corresponded to the helper MLV and was

![Figure 1](image-url)
infectious for NIH-3T3 cells. The other component may correspond to a VSV(RHV) pseudotype: it was infectious for all three human cell cultures used, but not for Vero or mink cells.

**Neutralization specificity of the VSV(RHV) pseudotype**

Finding a selective indicator cell system for detection of the VSV(RHV) pseudotype even in the presence of an excess of the VSV(MLV) pseudotype enabled us also to test its neutralization specificity.

Sheep antisera to antigens assembled from human cell lines HMB2, T47D and HeLa by VSV ts-O45 or by HVJ, a non-immune sheep serum (i.e. preimmune anti-HeLa) and a sheep anti-MLV serum were used (Table 1).

All the sera to antigens derived from human cells caused significant plaque reduction of the VSV(RHV) pseudotype. This observation suggested three possible interpretations: (i) specific neutralization of the VSV(RHV) pseudotype, (ii) blocking of a virus receptor on the surface of HeLa cells by antibodies to human cell antigens, or (iii) cross-reaction of sheep antibodies to human cell antigens (e.g. common oligosaccharide residues in cellular or viral glycoproteins) with related antigens synthesized by mouse cell enzymes and present on the viral surface.

Therefore, control neutralizations were included. The first of these included the same antisera, and the pseudotype VSV(X-MLV) produced in mink cells and assayed in HeLa cells. In this variant of the experiment, only the serum to HeLa cell-derived antigens caused a significant plaque reduction of VSV(X-MLV), most likely by blocking HeLa cell surface structures. Other sera, directed to antigens of human cell lines HMB2 or T47D, caused no significant plaque reduction. This reduced (except, to some extent, for anti-HeLa serum) the likelihood of explanation (ii).

In another control neutralization, VSV(MLV) pseudotype was produced and assayed in NIH-3T3 cells. None of the sera to human cell-derived antigens caused any detectable plaque reduction. This ruled out explanation (iii).

Sheep anti-MLV serum neutralized all three pseudotypes, VSV(MLV), VSV(X-MLV) and VSV(RHV), to a very similar extent.

Additional experiments (results not shown) included the same sheep sera to VSV-assembled human cell antigens, but pre-absorbed with an excess of VSV antigens as described by Závada et al. (1983). This absorption had no influence on the neutralization of the VSV(RHV) pseudotype.

**Immune cell aggregation**

The purpose of the very simple experiment shown in Fig. 2 was to find whether a human cell-related antigen could be detected directly on the surface of NIH-F4 cells, and if so, whether or not it was present on all of them. Suspensions of NIH-F4 cells, in parallel with HMB2 and NIH + MLV + MSV cells, were seeded in Petri dishes in growth medium with or without anti-HMB2 serum. The antisera prevented attachment and caused aggregation of HMB2 and NIH-F4 cells, but not of NIH + MLV + MSV cells. This result indicated that an antigen related to those of human cells was expressed on the surface of all the NIH-F4 cells.

In analogous experiments (not shown), performed in microplates (Linbro, 96 flat-bottomed wells), non-immune and anti-HMB2 sheep sera were titrated (twofold dilutions from 1:50 to 1:3200) with the same three cell cultures as above. These experiments included dilution series with and without complement. Both non-immune and anti-HMB2 sera diluted 1:50 interfered with attachment to all three cell lines. Anti-HMB2 dilutions from 1:100 to 1:800 aggregated HMB2 and NIH-F4 cells, but not NIH + MLV + MSV cells. Very similar results were obtained also with anti-HeLa and anti-T47D sera. Addition of complement to anti-HMB2 caused lysis of HMB2, but not of NIH-F4 cells.
Table 1. Neutralization of VSV pseudotypes with envelope antigens of RHV, X-MLV and MLV by immune sheep sera to host cell-derived antigens, selectively assembled into the virions of VSV ts-O45 or of HJV from human cell lines HMB2, HeLa or T47D, and by anti-MLV serum*

<table>
<thead>
<tr>
<th>Serum added</th>
<th>Reciprocal dilution</th>
<th>VSV(RHV), HeLa</th>
<th>VSV(X-MLV), HeLa</th>
<th>VSV(MLV), NIH-3T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>362 0</td>
<td>287 0</td>
<td>324 0</td>
</tr>
<tr>
<td>Non-immune</td>
<td>50</td>
<td>318 12</td>
<td>290 -1†</td>
<td>321 1</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>358 1</td>
<td>284 1</td>
<td>318 2</td>
</tr>
<tr>
<td>Anti-(VSV)HMB2</td>
<td>50</td>
<td>22 94</td>
<td>278 3</td>
<td>325 0</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>170 53</td>
<td>293 -2</td>
<td>330 -2</td>
</tr>
<tr>
<td>Anti-(HJV)HMB2</td>
<td>50</td>
<td>11 97</td>
<td>244 15</td>
<td>311 4</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>210 42</td>
<td>288 0</td>
<td>323 0</td>
</tr>
<tr>
<td>Anti-(VSV)HeLa</td>
<td>50</td>
<td>4 99</td>
<td>69 76</td>
<td>298 8</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>116 68</td>
<td>235 18</td>
<td>318 2</td>
</tr>
<tr>
<td>Anti-(VSV)T47D</td>
<td>50</td>
<td>138 62</td>
<td>281 2</td>
<td>340 -5</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>275 24</td>
<td>278 3</td>
<td>324 0</td>
</tr>
<tr>
<td>Anti-MLV</td>
<td>1000</td>
<td>7 98</td>
<td>40 86</td>
<td>3 99</td>
</tr>
<tr>
<td></td>
<td>4000</td>
<td>192 47</td>
<td>195 32</td>
<td>165 49</td>
</tr>
</tbody>
</table>

* Plaque counts are sums from three parallel Petri dish cultures. Reaction mixtures contained in 1 ml an expected 100 p.f.u. of pseudotype, sheep anti-VSV serum diluted 1 : 200 and the other sera in final dilutions given in the Table. After 1 h at 37 °C, the mixtures were directly plated for plaques at 1 ml/plate.
† Increases are expressed as negative values.

Radioimmunoprecipitation and PAGE of human cell surface proteins by antiserum to RHV antigens expressed in mouse cells

The experiments described above indicated that the NIH-F4 cells expressed an antigen(s) reacting with antibodies to VSV-assembled human cell proteins.

In the next experiment (Fig. 3) we tried to find the converse serological reaction. We immunized one group of mice with MLV antigens (from NIH + MLV + MSV cells), and the other with MLV and RHV antigens (from NIH-F4 cells). These two immune sera (pooled) were used for immunoprecipitation of 125I-labelled surface antigens of three human cell lines (HEC, HMB2 and HeLa). Anti-MLV did not precipitate any human cell proteins, but anti-(MLV + RHV) precipitated a protein of 85K mol. wt. from all three human cell lines. The significance of this observation was further supported by a comparison with total, unprecipitated labelled proteins, which in each of the three cell lines showed a rather different pattern.

DISCUSSION

The results presented in this paper appear to us to be compatible with the explanation that the recovered agent, provisionally termed RHV, might be an endogenous human retrovirus. However, conclusive identification of this apparently new agent will require further study.

One of the distinctive features of the apparent VSV(RHV) pseudotype is its infectivity for three human cell lines, and a simultaneous lack of infectivity for Vero or mink cells (Fig. 1). This contrasts with the host range spectra described for VSV pseudotypes having envelope antigens provided by the following oncoviruses: xenotropic mouse leukaemia and mammary tumour viruses (Závada et al., 1977), primate and feline type C viruses (Schnitzer et al., 1977), and bovine leukaemia virus (Závada et al., 1979). For all of these, Vero and/or mink cells represent the most sensitive assay system. Also VSV pseudotypes with envelope antigens of the human T cell leukaemia retroviruses HTLV-1 and HTLV-2 are infectious both for human and for mink cells (Clapham et al., 1984). The VSV(LAV-1/HTLV-III) pseudotype is infectious only in human cells that express the CD4 (T4) antigen; it is not infectious for HeLa cells (Dalgleish et al., 1984). Thus, RHV determines a host range specificity for VSV pseudotypes which is distinct from any of these animal or human retroviruses. The observed host range of VSV(RHV) is consistent with the view that RHV might be a new human ecotropic virus.
Rescued human virus

Fig. 3. Immunoprecipitation and PAGE of human cell surface proteins labelled with $^{125}$I. Extracts were prepared from HEC (lanes 1, 4 and 5), HMB2 (lanes 2, 6, and 7) and HeLa (lanes 3, 8 and 9) cells. Lanes 1, 2, and 3, total, non-precipitated proteins; lanes 4, 6 and 8, proteins immunoprecipitated with anti-MLV serum; lanes 5, 7 and 9, proteins immunoprecipitated with anti-(MLV + RHV) serum. Electrophoresis was in an 8% gel.

Neutralization of the VSV(RHV) pseudotype by antisera to VSV-assembled human cell proteins (Table 1) further suggests the human origin of RHV. However, these sera contain antibodies to several (or many) different proteins of the surface of human cells, and in other types of reactions they show higher titres than in neutralization of VSV(RHV) (Závada et al., 1983; Závada & Huang, 1984). Thus, only a minor component of their antibody spectrum seems to be responsible for neutralization of VSV(RHV). Using these antisera in radioimmunoprecipitation and PAGE of $^{125}$I-labelled NIH-F4 cells, we found no specific protein band on autoradiographs of the gels (result not shown); this could perhaps be explained by a low concentration of RHV antigen in NIH-F4 cells. Provided the titre of VSV pseudotypes is proportional to the concentration of available envelope proteins, then from Fig. 1 we would expect a 300-fold lower amount of RHV protein than MLV gp70; if so, it may have escaped detection on the autoradiograph.

Neutralization of the VSV(RHV) pseudotype by anti-MLV serum (Table 1) most probably means that the envelope of these pseudotype virions is a mosaic, composed of antigens corresponding to those of RHV, MLV and VSV. Reaction of MLV gp70 with antibody
molecules may sterically block the RHV antigen (Weiss & Bennett, 1980; Závada, 1982). Alternatively, both RHV and MLV determinants might be necessary for infection of human cells, and neutralization of either would inhibit the infectivity of a mosaic VSV pseudotype. This would apply also if the RHV protein is not fully functioning and is responsible only for attachment to specific human cell receptors, whereas the MLV protein is involved in the subsequent steps of penetration.

Mouse anti-(MLV + RHV) serum precipitated a protein of 85K from all three human cell lines (Fig. 3). This resembles a 88K protein previously described by Závada & Huang (1984) as a VSV-assembled protein common to different cell lines and precipitated by all three antisera. The small difference of $M_r$ could be accounted for by differences in the sets of reference proteins used on the two occasions. Such a molecular weight would correspond approximately to that of the uncleaved precursor $env$ protein of known mammalian type C viruses (Dickson et al., 1984).

At present, the biological significance of RHV is entirely unknown. The neutralization of the VSV(RHV) pseudotype by antisera to antigens derived from HMB2, T47D and HeLa cells and the finding of a very similar 85K protein band precipitated by mouse anti-RHV serum in normal, low-passage human fibroblasts (HEC) and in tumour cell lines (HMB2 and HeLa) indicate that RHV-related proteins may be common to other kinds of human cells.

However, there is a hint that RHV might in fact possess an interesting biological activity: from the beginning, we noticed a strikingly different morphology of NIH-F4 cells compared to parallel cultures of NIH + MLV + MSV cells. This can be seen in Fig. 2(c, e). While the NIH + MLV + MSV cells are highly refractile, spindle-shaped and separated from each other, NIH-F4 are more flattened, less refractile and tend to stick to each other. In dense, old cultures NIH + MLV + MSV cells always detach from glass or plastic as individual cells, but NIH-F4 as whole sheets. The first impression is that RHV somehow weakens the transformation induced by MSV. This conspicuous difference was reproducible in all experiments on parallel transmission of MSV and MLV and of MSV, MLV and RHV complexes to normal NIH-3T3 cells, both when using medium filtrates or co-cultivation with virus donor cells treated with mitomycin C. On the other hand, in focus-formation assays for MSV, both the focus titres and their morphology were indistinguishable between viruses from the two sources. Thus, the Harvey MSV appears to be identical in both types of cultures, and it can be separated from RHV by dilution. This modification of MSV-induced transformation by RHV will require a separate thorough study.

At the moment, there is no direct suggestion of RHV being harmful to man, but in spite of this, it should be handled as potentially biohazardous for these theoretical reasons: first, RHV has a human tropism, and thus it might facilitate infection of human cells by oncogenic Moloney MLV and Harvey MSV by phenotypic mixing; second, RHV might be an oncogenic recombinant of MLV or MSV with endogenous human retrovirus sequences; third, RHV has been derived from human melanoma cells, and its possible role in oncogenesis cannot be ruled out.

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REFERENCES


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