Acta virol. **27**: 110—118, 1983

VESICULAR STOMATITIS VIRUS PHENOTYPICALLY MIXED WITH RETROVIRUSES: AN EFFICIENT DETECTION METHOD

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Received June 29, 1982

Summary. — Two methods of assaying vesicular stomatitis virus (VSV) particles phenotypically mixed with retrovirus-coded antigens were compared. Each of them detected phenotypically mixed particles with different minimum proportion of surface glycoprotein molecules of the donor virus, and consequently also profoundly different proportions of VSV virions containing retrovirus antigens. Only a low proportion (10^{-4}) of VSV virions grown in XMuLV-infected rabbit SIRC cells behaved as pseudotypes, resistant to anti-VSV serum and neutralized by anti-XMuLV serum. VSV produced in mouse L cells did not contain significant titre of pseudotype particles in the neutralization test. However, when immunoprecipitation was used with corresponding antibody and *Staphylococcus aureus* cells, almost 100% of the VSV virions produced in L cells and in XMuLV-preinfected SIRC cells were found to contain MuLV-related antigen molecules.

Key words: *rhabdovirus, retrovirus, phenotypic mixing, pseudotype*

Introduction

Phenotypic mixing between various enveloped viruses often occurs when such viruses infect the same cell (for reviews see Závada, 1976, 1977, 1982; Boettiger, 1979; Weiss, 1981). However, in some cases of such dual infections phenotypic mixing has not been detected (Závada, 1982). This might be due to the method used, which detects only virions, behaving as pseudotypes. These are particles resistant to antibodies directed against the virus which provides the genome (e.g. VSV) of the mixed particle, but are sensitive to antiserum directed against the virus which provides the coat antigens (e.g. retrovirus). Such pseudotypes are probably covered by glycoprotein (gp) of the donor virus and contain only a low proportion of VSV spikes (Weiss and Bennett, 1980; Závadová and Závada, 1980).

However, phenotypically mixed particles with a prevalence of VSV-gp in the coats would escape detection when the virus neutralization is used to assay for pseudotype formation. Such mixed virions can be identified by

several other techniques; a most efficient method has been adopted from radioimmunoassay, employing fixed *Staphylococcus aureus* cells. Antiserum to the gp, which is being tested for, is first added to the virus suspension from mixedly infected culture, followed by the addition of *Staphylococcus aureus* bearing the A protein. The virus-antibody complexes bind to *S. aureus* cells and can be pelleted at low speed centrifugation. The supernate is assayed for remaining infectivity (Weiss and Bennett, 1980). These authors used in their experiments VSV in combination with RAV-1, which is a very efficient donor of gp for pseudotype particles — about 10% of all VSV virions are rendered resistant to anti-VSV serum. Thus it was no surprise that almost 100% of VSV virions in the same population were found to be phenotypically mixed, when using the more sensitive test.

The purpose of the present work was to use the *S. aureus* method in order to establish an actual level of phenotypic mixing of VSV when grown in the presence of retroviruses, which form pseudotypes with VSV (resistant to anti-VSV serum) much less efficiently than RAV-1. For this purpose we chose XMuLV-infected rabbit SIRC cells, where the frequency of VSV pseudotypes is only about 10^{-4}, and mouse L cells, which appear to be pseudotype-negative. The L cells produce type-C virions and contain MuLV--related antigens (Kindig *et al.*, 1968; Schäfer *et al.*, 1972), including the gp70 (Russ *et al.*, 1983). In this paper we demonstrate that the VSV progeny of both L cells and XMuLV-infected SIRC cells contains virtually 100% of phenotypically mixed VSV virions, when the *S. aureus* method is being used.

Materials and Methods

Viruses, media, antisera, assay conditions and the pseudotype test. These were similar to those described before (Závada *et al.*, 1977, 1978, 1979). In addition, the L cells (A9 clone), obtained from Dr. M. Fried, London, were used.

Anti-XMuLV serum was prepared by immunizing a rabbit with XMuLV, reproduced in human embryo fibroblasts and purified by sucrose gradient centrifugation. The rabbit was given three immunizing doses, each of approx. 0.2 mg protein of purified virus; the antigen was suspended in complete (first dose) or incomplete (other two doses) Freund's adjuvant and injected intramuscularly. The intervals between immunization doses were 6 weeks, the rabbit was bled 7 days after the third dose. This serum showed very similar neutralization titres both for VSV(Mo-MuLV) and for VSV(XMuLV) pseudotypes.

Absorption of "non-specific" antibodies from this serum. 5 ml of the serum diluted 1 : 10 were absorbed twice with a pellet of VSV-infected mink cells. The cells were pelleted following a complete CPE by VSV and each pellet contained approximately 10^8 such cells. Subsequently, the serum was absorbed to non-infected HeLa and NIH-3T3 cells (approx. 5×10^7 of each). Each absorption proceeded for 60 min on ice, with occasional shaking. After the final centrifugation, the serum was heated for 20 min at 56 °C to inactivate infectious VSV.

Immunoprecipitation of virions by antibody and S. aureus cells was essentially similar to the procedure used by Weiss and Bennett (1980). 50 µl containing 160 PFU of VSV were mixed with 50 µl of serum and incubated at room temperature for 30 min. Subsequently 1.5 ml of suspension of *S. aureus* cells (0.5% w/v) was added, and further incubated for 60 min. Finally, the mixtures were centrifuged at 3500 rev/min for 30 min and 1 ml aliquots of the supernates were plated for plaques on confluent cultures of mink CCL/64 cells seeded in Petri dishes.

Inhibition of immunoprecipitation with cell culture extracts. 50 µl of diluted sera were first mixed with 50 µl of cell extracts. Following 30 min of incubation, 50 µl of virus suspension was added. This was followed by the addition of *S. aureus* as described above. The cell extracts were

Table 1. Failure to demonstrate VSV pseudotype production in mouse L cells

VSV grown in cells:	Time of harvest (hr)	Assay cells	Antiserum treatment			
			None	Anti-XMuLV	Anti-VSV	Anti-VSV + anti-XMuLV
NIH	8	NIH	7.43*	7.39	< 1.00	< 1.00
		Vero	8.51	8.55	1.48	1.00
NIH + MoMuLV	8	NIH	7.45	7.38	5.21	< 1.00
		Vero	8.42	8.51	1.30	1.48
SIRC	8	NIH	6.50	6.48	< 1.00	< 1.00
		Vero	7.42	7.35	< 1.00	< 1.00
SIRC + XMuLV	8	NIH	7.25	7.25	1.30	< 1.00
		Vero	8.30	8.22	4.29	1.30
L	3	Vero	4.42	3.80	< 1.00	< 1.00
	4	Vero	5.20	4.95	< 1.00	< 1.00
	8	NIH	6.80	6.67	1.00	< 1.00
		Vero	7.74	7.65	1.30	1.48

* Virus titres calculated for undiluted cell culture fluids (log PFU/ml).
Reacting mixtures contained infectious cell culture fluids 1 : 10 plus no antiserum, anti-VSV serum (1 : 50), anti-XMuLV serum (1 : 10) or both. The mixtures were incubated at 37 °C for 60 min, subsequently diluted and plated.

prepared by sonication of cell suspensions (in saline) with the Raytheon sonic oscillator (10 kc, 150 w) for 4 min. The extracts were clarified by centrifugation at 3000 rev/min for 15 min and protein contents determined according to Lowry.

All dilutions of viruses, sera, antigens and *S. aureus* were prepared on complete Dulbecco PBS with 1% of foetal calf serum (FCS). All immune sera as well as sera added to the media were heated for 30 min at 56 °C. All virus stocks were prepared in Basal Eagle's medium containing 5% of foetal calf serum, which has been added to the cell cultures after infection.

Results

Failure to demonstrate VSV pseudotype particles with MuLV-related antigens in mouse L cells

As seen in Table 1, VSV reproduced in mouse L cells did not show any detectable proportion of pseudotype resistant to anti-VSV serum. On the other hand, when VSV was grown in Mo-MuLV-infected mouse NIH-3T3 cells or in XMuLV-infected rabbit SIRC cells (included as positive controls), pseudotype titres similar to those reported before were detected (Závada *et al.*, 1977). To increase the chance of detecting VSV virions, which could be neutralized both by anti-VSV as well as anti-XMuLV sera, we harvested VSV from L cells also after only 3 or 4 hr post infection. The rationale was that most efficient phenotypic mixing takes place at early intervals (Witte and Baltimore, 1977). Indeed, VSV harvested early after infection contained a significant proportion (about 40—60%) of virions carrying the VSV genome, which behaved as doubly-neutralizable virus.

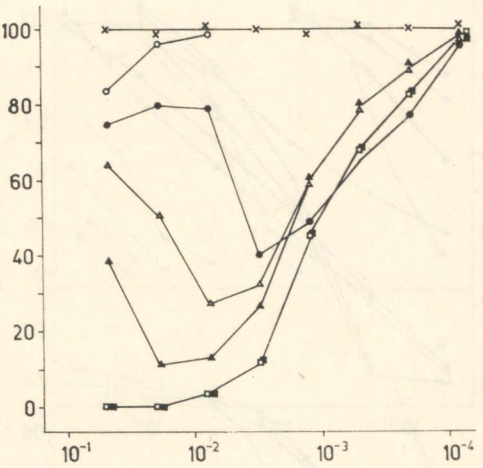

Fig. 1.

Determination of optimum *S. aureus* concentration

160 PFU of VSV harvested 3 hr after infection of L cells (in 0.05 ml) were incubated with 0.05 ml of anti-XMuLV serum (or non-immune rabbit serum) for 30 min. Subsequently 1.5 ml of *S. aureus* cell suspension was added and further incubated for 60 min. Finally, the mixtures were centrifuged, and 1 ml aliquots of the supernatants were plated for plaques.

The w/v concentrations of *S. aureus* were: 0% (○), 0.05% (●), 0.1% (△), 0.2% (▲), 0.5% (□), 1% (■) in combination with anti-XMuLV serum. The 0.5% *S. aureus* concentration (X) was also used in combination with control rabbit serum. Abscissa: dilutions of sera in reacting mixtures before addition of *S. aureus* ($\log_{10}$ values); ordinate: per cent of the original VSV infectivity remaining in the supernatant.

Immunoprecipitation of VSV reproduced in L cells by antibodies to XMuLV and S. aureus cells

The purpose of the experiment shown in Fig. 1 was to find the range of suitable concentrations of anti-XMuLV- serum and of *S. aureus* cells for detection of phenotypically mixed virions. The results shows that the titration curve of anti-XMuLV serum dilutions between 1 : 50 — 1 : 10 000 covers 0 — 100% reaction with VSV reproduced in L cells. A 0,5% concentration of *S. aureus* cell suspension was sufficient to cover these dilutions of antiserum. Non-immune rabbit serum, also included in this experiment, showed no reaction with VSV reproduced in L cells, as detected by subsequent addition of *S. aureus* and centrifugation.

The specificity of this reaction was tested and improved in experiment outlined in Fig. 2. The immune rabbit serum was expected to contain, besides antibodies specific for XMuLV antigens, also antibodies reacting with cells-specific antigenic substances (oligosaccharidic chains, glycolipids etc.) and antigenic lipids acquired from calf serum used in growth media, which had been present in XMuLV virions used for immunization. In addition, also broadly reactive antibodies could have been raised by complete Freund's adjuvant. These „non-specific" antibodies might interfere in the test, since VSV virions also contain lipidic and oligosaccharidic antigens acquired from the host cells and from the growth medium. Therefore, we tried to absorb the immune serum with „normal antigens" as described in Materials and Methods.

Unabsorbed anti-XMuLV serum showed some non-specific reaction with VSV grown in MuLV-negative cells (NIT-3T3 and SIRC); this reaction was satisfactorily (but not completely) abrogated by the absorption procedure

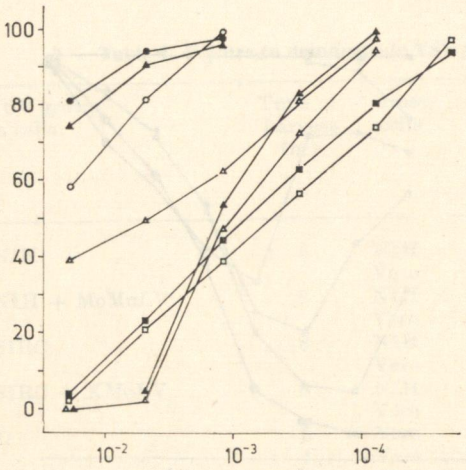

Fig. 2.

Absorption of non-specific antibodies from anti-XMuLV serum

VSV was grown for 3 hr in following cells: ○ SIRC, □ SIRC+XMuLV, △ NIH, △ L.

Open symbols — unabsorbed serum; full symbols — serum absorbed with "normal antigens" (see Methods). For details see legend to Fig. 1.

used. At the same time, this absorption of „non-specific" antibodies from the serum had very little effect on its reaction with VSV grown in L or in SIRC + XMuLV cells, thus showing the prevalence of antibodies specific for MuLV-related antigens as detected by the reaction used. The titration curve for antiserum reaction with VSV produced in L cells was somewhat steeper than for VSV grown in SIRC + XMuLV cells.

Effect of the time of harvest of phenotypically mixed VSV on the immunoprecipitation with XMuLV-specific antibody

VSV harvested at early intervals following infection appears to contain more MuLV-related antigens when compared to virus harvested after pro-

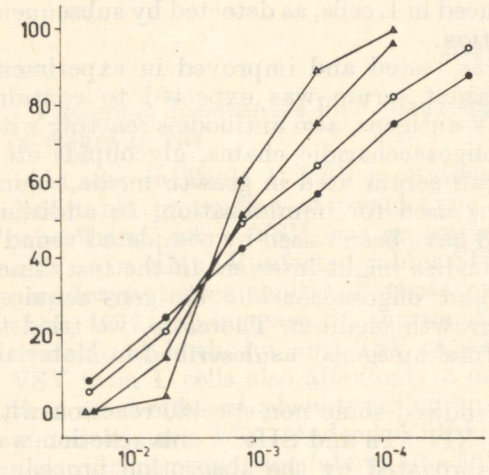

Fig. 3.

Effect of the time of harvesting VSV on the reaction with anti-XMuLV serum and *S. aureus* cells

VSV harvested from SIRC+MuLV cells 3 hr (○) or 8 hr (●) after infection; VSV harvested from L cells after 3 hr (△) or 8 hr (▲). Anti-XMuLV serum was absorbed with "normal antigens." For details see legend to Fig. 1.

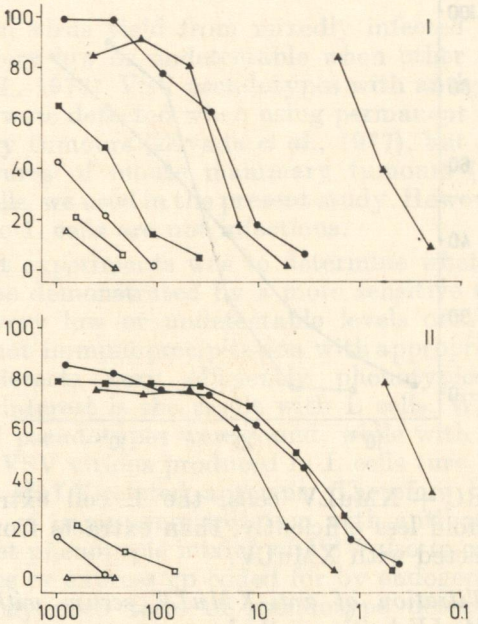

Fig. 4.

Absorption of MuLV-specific antibodies with cell extracts and with purified XMuLV

0.05 ml of anti-XMuLV serum diluted 1 : 50 (pre-absorbed with "normal antigens") was allowed to react for 30 min with 0.05 ml of cell extracts. Subsequently, 160 PFU of VSV in 0.05 ml were added and further incubated for 30 min. Finally, 1.5 ml of 0.5% *S. aureus* was added and residual infectivity tested as in Fig. 1. I — VSV grown for 3 hr in SIRC + XMuLV cells; II — VSV grown in L cells. Absorbing antigens: extracts from ○ SIRC, ● SIRC+MXuLV, △ LKC, ▲ LKC+XMuLV, □ HIH, ■ L cells, ▲———▲ most in the right: gradient-purified XMuLV (reproduced in mink cells).

Abscissa: μg protein/0.05 ml; ordinate: per cent of residual infectivity.

longed incubation, as can be seen in Table 1. Therefore, we tested the effect of the interval of harvesting the virus from SIRC + XMuLV and from L cells on the proportion of VSV virions containing MuLV-related antigens, using precipitation with antibody and *S. aureus* cells. Fig. 3 shows, surprisingly, that the various intervals of virus harvest had almost no effect on the titration curves of the anti-XMuLV serum with VSV reproduced in cells containing MuLV-related antigens. Apparently, the vast majority of VSV virions obtained from these cells 3 or 8 hr after infection contained at least some MuLV antigens.

Absorption of XMuLV-specific antibodies with cell extracts containing MuLV-related antigens

The reaction of phenotypically mixed VSV with antibodies to XMuLV and *S. aureus* can also be employed for detection and titration of MuLV--related antigens in crude cell extracts (Fig. 4). The extracts from cells infected with XMuLV and from L cells absorb the antibodies, which bind to phenotypically mixed VSV, while extracts from corresponding control cells inhibit this reaction only when used in extremely high concentration (presumably a non-specific reaction).

In addition, this experiment shows that the antigens present in L cells are related to, but not identical with those in XMuLV-infected SIRC or LKC cells. The extracts from all these three cell lines absorb very similarly antibodies binding to VSV from L cells. In contrast, for VSV reproduced in

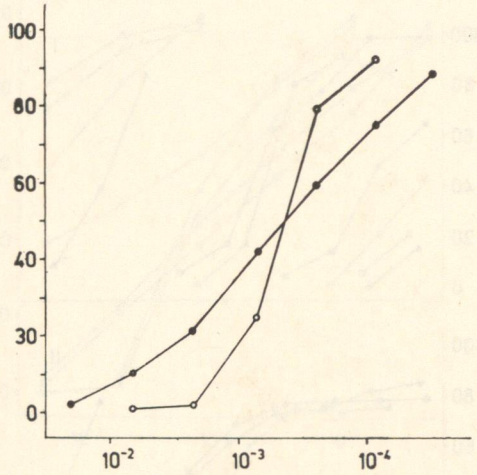

Fig. 5.

Comparison of titration curves obtained by two methods

○ = neutralization of VSV (XMuLV) reproduced in SIRC+MuLV cells with anti-XMuLV serum (= fraction of infectivity resistant to anti- VSV serum)

● = immunoprecipitation of total VSV reproduced in SIRC+XMuLV with anti-XMuLV serum and *S. aureus* cells (0.5%). For coordinates see legend to Fig. 1.

SIRC + XMuLV cells, the L cell extract absorbs the antibodies about 10-fold less efficiently, than extracts from SIRC or LKC cells productively infected with XMuLV.

Titration of anti-XMuLV serum with VSV, phenotypically mixed with XMuLV by two methods

A comparative titration by two methods of anti-XMuLV serum with VSV reproduced in SIRC + XMuLV cells is shown in Fig. 5. Method I was neutralization of infectivity of the VSV (XMuLV) pseudotype. Method II was immunoprecipitation using *S. aureus* cells. In spite of profound differences between the two tests, both showed nearly the same 50% end-point titre of antibodies. The slope of titration curve II was lower, indicating probably a higher heterogeneity of phenotypically mixed virions as well as of types of antibodies involved. Conceivably, in this latter test participate also non-neutralizing antibodies.

Discussion

The frequency of VSV pseudotypes with surface antigens provided by unrelated enveloped viruses varies within extremely wide limits. It depends both on the virus used as a donor of surface antigens and on the type of cells, which were mixedly infected. The proportion of VSV pseudotypes in total VSV infectivity was reported to be as high as 50% in combination with herpes simplex virus (Huang *et al.*, 1974), and 10% with avian retrovirus RAV-1 (Love and Weiss, 1974). On the other hand, when xenotropic murine leukaemia or mouse mammary tumour viruses were used, it was as low as 10^{-4} or 10^{-5} (Závada *et al.*, 1977).

In some instances the VSV(retrovirus) pseudotypes could not be detected in spite of the fact that infectious retrovirus particles were produced in the cultures used. VSV pseudotypes with surface antigens of primate type C

retroviruses were easily detected in virus yield from mixedly infected bat Tb-1-lu cells, but their titre was very low or undetectable when other cell lines were employed (Schnitzer *et al.*, 1977). VSV pseudotypes with antigens of murine mammary tumour virus were detected when using permanent cell lines derived from mouse mammary tumours (Závada *et al.*, 1977), but not in MMTV-producing primary cultures of mouse mammary tumours (unpublished). A similar case is the L cells, we used in the present study. However, the C type virions produced by the L cells are not infectious.

The main purpose of the present experiments was to determine whether phenotypic mixing of VSV could be demonstrated by a more sensitive test in mixed infections which yield very low or undetectable levels of VSV pseudotypes. Our results showed that immunoprecipitation with appropriate antibodies and *S. aureus* cells detects very efficiently phenotypically mixed VSV virions. Of particular interest is the result with L cells. When anti-VSV serum was used, no VSV pseudotypes were found, while with the present method virtually 100% of VSV virions produced in L cells turn out to be phenotypically mixed with MuLV-related antigens. Therefore it is reasonable to assume that the use of immunoprecipitation with antibodies and *S. aureus* cells is likely to detect phenotypic mixing of VSV also in cells, which are infected with retroviruses or express gp coded for by endogenous proviruses, in which the previously used tests of pseudotype detection proved negative or dubious.

There may be several reasons for the failure to detect infectious VSV pseudotypes in L cells: low concentration of the gp of the donor retrovirus, lack of known susceptible indicator cell culture, imperfect processing of the gp coded for by vertically transmitted proviruses. In the last case, the gp may not be fully functional so as to be enable the first steps of the infection process. In a parallel study, the cells were surface-labelled with ^{125}I and their extracts were analysed by immunoprecipitation and polyacrylamide gel electrophoresis (Russ *et al.*, 1983). These experiments showed that in L cells a relatively lower amount of MuLV-related antigens was labelled with ^{125}I than in rabbit (SIRC) or in lamb (LKC) cells productively infected with XMuLV. Moreover, in L cells a significant amount of larger MuLV-related molecules of approximately 90K was detected. These 90K molecules were also assembled into VSV virions reproduced in L cells. These 90K molecules might be the precursor molecules coded for by the *env* gene of vertically transmitted type C virus. In contrast, the XMuLV-infected SIRC and LKC cells shed virus particles which contain only the gp70 molecules. The results presented in this paper may provide an explanation for the failure to detect VSV(MuLV) pseudotypes in L cells, when the serum neutralization tests are used.

An additional advantage of the immunoprecipitation with antibodies and *S. aureus* cells is that it might detect non-neutralizing antibodies, which can bind to viral surface structures.

Acknowledgements. The authors greatly appreciate the comments and kind help of Dr. Abraham Karpas in preparing this manuscript and the skilful technical assistance of Miss K. Rozborilová.

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