Phenotypic Mixing between Two Primate Oncoviruses

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SUMMARY

Phenotypic mixing between the primate oncoviruses HL23V and BEV has been demonstrated to occur in doubly-infected bat lung (Tb) cells with the production of HL23V(BEV) pseudotype virus. The presence of the HL23V(BEV) pseudotype permitted the host range for replication of HL23V to be extended to murine cells previously ‘resistant’ to HL23V replication due to a block at the level of virus penetration. Expression of BEV genetic information was observed in doubly-infected rat cells and also in mouse and rat MSV-transformed non-producer cell lines co-cultivated with BEV-producing Tb cells. No evidence for genetic recombination between these viruses could be demonstrated.

Phenotypic mixing between oncoviruses and other enveloped viruses (Zavada, 1976) as well as between oncoviruses themselves (Zavada, 1976; Besmer & Baltimore, 1977; Ishimoto et al. 1977; Levy, 1977a, b; Weiss & Lo-Wong, 1977) has been demonstrated in vitro and postulated to be of pathogenic significance in vivo (Ishimoto et al. 1977). The acquisition of novel envelope antigens by a superinfecting virus may result in a markedly altered host range for penetration permitting the introduction and possible replication of the virus genome in cells otherwise nonpermissive to these viruses (Besmer & Baltimore, 1977; Ishimoto et al. 1977; Levy, 1977a, b; Weiss & Lo-Wong, 1977).

During recent years, various investigators have independently reported the isolation from human tissues of biologically active C-type viruses (Panem et al. 1971; Gabelman et al. 1975; Gallagher & Gallo 1975; Nooter et al. 1975, 1977; Teich et al. 1975). In all cases one of the components has been shown to be closely related to simian sarcoma-associated virus (SSAV). However, in the two cases examined most closely (Teich et al. 1975; Prochownik & Kirsten, 1976; Reitz et al. 1976) evidence for a second, distinct oncovirus, identical with or closely related to the baboon endogenous virus (BEV), has repeatedly been found. The fact that these same two oncoviruses have rarely, but on independent occasions, been demonstrated together in human tissue suggests that the replication of these viruses in vivo may be a consequence of some necessary interactions between the two agents. We have pursued in vitro investigations to elucidate possible mechanisms for such interactions.

The viruses studied, the SSAV-like component (Teich et al. 1975) of the HL23V virus complex (HL23V; Gallagher & Gallo, 1975) and the M7 strain of baboon endogenous virus (BEV) have been previously characterized. Canine thymus cells (8155) were provided by G. Peries (Université Paris VII, Hôpital St Louis, Paris, France), MSV-infected non-producer mink cells (Mink S' L); Henderson et al. 1974) by N. Teich (Imperial Cancer Research Fund, London, England) and MSV-infected nonproducer Balb/3T3 cells (KA31) by B. Ozanne (University of Texas Southwestern Medical School, Dallas, Texas). The origin and maintenance conditions of all other cells have been described previously (Schnitzer et al. 1977).

All infections were initiated in the presence of Polybrene (20 μg/ml) except the XC assay.
which was performed using 7605L cells pre-treated with DEAE-dextran as previously described (Rowe et al. 1970). To establish doubly-infected cells, rapidly dividing cell cultures were separately infected with each virus, passaged until reverse transcriptase activity was detectable in the supernatant and then superinfected with the second virus. Progeny virus was harvested from doubly-infected cells after three to five further passages. Reverse transcriptase activity was measured and focus assays performed according to standard conditions (Teich et al. 1975; Weiss & Lo-Wong, 1977). Virus neutralizations were performed by incubating 10 µl of virus with 90 µl of the appropriate heat-inactivated antiserum (Schnitzer et al. 1977) for 2 h at room temperature. Serial dilutions were then inoculated on to cell monolayers and allowed to adsorb in the presence of 20 µl/ml of Polybrene for 1 h after which the inoculum was removed and the cells washed twice with medium to remove residual antiserum.

To examine possible interactions between BEV and HL23V, investigations initially concentrated on searching for pseudotype virions among progeny released from doubly-infected bat lung (Tb) cells, a line supporting replication of both BEV and HL23V. Progeny virus was harvested from cells infected with only BEV or HL23V and from doubly-infected cells. As HL23V is an XC plaque-forming virus (Teich et al. 1975), pseudotype virus with the composition HL23V(BEV) could be identified by means of a standard XC assay and distinguished from HL23V itself by both the use of appropriate antisera and by exploiting differences in host range (Schnitzer et al. 1977) – vide infra. Regardless of the original order of virus infection, doubly-infected cells regularly released XC plaque-forming virus not able to be neutralized by anti-SSAV serum alone but completely neutralized when both anti-SSAV and anti-BEV serum were used together (Table 1A). The relative proportion of HL23V(BEV) pseudotype to HL23V found in the supernatant varied from 0.1% to 10%, apparently depending on the condition and passage level of the cells, the highest proportion being seen within a few passages of superinfection. The reciprocal pseudotypes with the composition BEV(HL23V) were not assayed due to lack of adequate amounts of antisera.

To investigate the extension of host range of HL23V(BEV) pseudotypes, an infectious centre (IC) XC assay was established which exploited the previously reported differences in host range to penetration of HL23V and BEV (Schnitzer et al. 1977). Rapidly dividing cells permissive (Rat-1, Tb) and resistant (NIH/3T3) to penetration by HL23V (Schnitzer et al. 1977) were infected with progeny virus from singly and doubly-infected cells and 24 h later were washed twice with glycine-HCl buffer pH 2.2 to remove any residual input virus glycoproteins (Steck & Rubin, 1966) from the cell surface. Following incubation for a further 48 h, supernatant virus was harvested, and the cells were then treated with mitomycin C 10 µg/ml for 2 h, trypsinized and inoculated on to a monolayer of 7605L cells, after which standard XC assay procedures were followed (Rowe et al. 1970). As shown in Table 1B, HL23V with its own coat would penetrate and replicate in Rat-1 and Tb cells and all progeny virus could be neutralized with anti-SSAV serum. No XC plaque-forming virus was found when NIH/3T3 cells were infected, presumably due to the block to replication at the level of virus penetration of the murine cells (Schnitzer et al. 1977). This interpretation was supported by the failure to detect HL23V-infected NIH cells by the IC XC assay. After infection of Tb and Rat-1 cell cultures with virus released from doubly-infected cells, all the cells could be shown to be producing XC plaque-forming virus, a result expected simply on the basis of HL23V alone in the virus inoculum. Infection of 'resistant' NIH/3T3 cells with identical inoculum resulted in a proportion of the cells giving rise to XC plaques in the IC XC assay. The ability to produce NIH/3T3 ICs was not altered by pre-treatment of the infecting inoculum with anti-SSAV serum but could be completely prevented by use of anti-
**Table I. Demonstration of host range for replication of HL23V by XC plaque assay**

<table>
<thead>
<tr>
<th>Inoculum virus from Tb cells infected with</th>
<th>Titre of XC plaque-forming virus*</th>
<th>Antiserum treatment</th>
<th>Cells infected</th>
<th>Infectious centres†</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>none</td>
<td>anti-SSAV+</td>
<td>anti-BEV</td>
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<tr>
<td>A. BEV + HL23V</td>
<td></td>
<td>5.6</td>
<td>4.4</td>
<td>5.7</td>
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<tr>
<td>HL23V</td>
<td></td>
<td>5.4</td>
<td>&lt; 2.0</td>
<td>5.6</td>
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<tr>
<td>BEV</td>
<td></td>
<td>&lt; 2.0</td>
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<tr>
<td>B. HL23V</td>
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<td>&lt; 2.0</td>
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<td>&lt; 2.0</td>
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<td>6.1</td>
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<tr>
<td>BEV + HL23V</td>
<td></td>
<td>4.0</td>
<td>&lt; 2.0</td>
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<td></td>
<td></td>
<td>3.3</td>
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<td>6.7</td>
<td>4.0</td>
<td>&lt; 2.0</td>
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<td></td>
<td></td>
<td>5.5</td>
<td>&lt; 2.0</td>
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<tr>
<td>BEV</td>
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* Log₁₀ XC p.f.u./ml on 7605L cells.
† % cells seeded.
‡ Not done.

SSAV serum plus anti-BEV serum, suggesting that the introduction of the HL23V genome into the NIH/3T3 cells occurred by means of the HL23V(BEV) pseudotypes (data not shown).

The identity of the XC syncytium-inducing virus released from NIH/3T3 cells was confirmed by the demonstration of complete neutralization of XC plaque-forming activity after treatment with anti-SSAV serum. The fact that such XC plaque-forming virus was released from NIH/3T3 cells, a line resistant to penetration by this virus, demonstrated that there was no absolute intracellular block to replication of this agent in murine cells. Indeed, once infection had been established, cells continued to release virus for up to 6 months upon continuous passage (data not shown). The magnitude of virus production, assayed by either XC plaque formation or reverse transcriptase activity, was not as high as usually observed in cell lines permissive for virus penetration, suggesting that some relative intracellular block to replication, as seen with xenotropic murine leukaemia virus (MULV-X) in murine cells (Levy, 1977a) might still exist.

In the course of examining the extension of HL23V host range, it was unexpectedly discovered that a proportion of progeny virus from rat cells infected with virus released from doubly-infected Tb cells appeared to be resistant to neutralization by anti-SSAV serum but was completely neutralized by additional treatment with anti-BEV serum (Table 1B). This finding strongly suggests that BEV replication was occurring in rat cells with the production of HL23V(BEV) pseudotypes under these conditions. Although BEV can penetrate rat or mouse cells (Schnitzer et al. 1977) it has previously been shown to be incapable of replication in these cell lines (Todaro et al. 1976). The possibility that HL23V(BEV) pseudotype production was a consequence of re-utilization of input virus glycoprotein was diminished by
the prior treatment of the doubly-infected rat cells with glycine buffer, pH 2.2, a method shown to be effective in removing avian (Steck & Rubin, 1966) and mammalian oncovirus surface glycoproteins (unpublished data).

To investigate further the possibility that BEV replication was occurring in rat cells and to assess the importance of the presence of HL23V for this replication, KNRK cells and KNRK cells previously infected with HL23V were co-cultivated with BEV-producing bat lung cells (Tb-BEV). To rule out the possibility of the release of an endogenous bat virus from the Tb-infected cells, another BEV-infected cell line believed to be free of endogenous oncoviruses, dog thymus (8155-BEV), was also utilized. Parallel cultures employing a mink nonproducer cell line, Mink S+L-, known to be permissive for both BEV and HL23V replication, were also established. Replication of BEV was assessed by the demonstration of sarcoma virus rescue in focus assays employing NIH cells, thereby selectively measuring production of MSV(BEV) in the case of the mixed infection. These results, shown in Table 2, demonstrate that effective rescue of transforming virus did occur from both the ‘permissive’ mink cells and the ‘resistant’ rat cells. The co-replication of HL23V in the rat cells appeared not to be necessary for sarcoma virus rescue, although higher focus-forming titres were consistently observed from cells previously infected with this virus. Studies in which KA31 cells, a mouse nonproducer cell line, were co-cultivated with Tb cells releasing BEV gave results similar to those with the KNRK cells. Experiments utilizing anti-SSAV and anti-BEV serum to neutralize the rescued MSV pseudotypes confirmed the fact that endogenous viruses were not responsible for the results obtained (data not shown).

Attempts to demonstrate pseudotype formation or sarcoma virus rescue by direct infection of HL23V infected Rat-1 cells or nonproducer cells, respectively, with BEV, rather than by co-cultivation with productively infected cells, were not successful, perhaps as a consequence of the lower amounts of virus present, as suggested by Levy (1977a). Alternatively, actual replication of BEV in mouse or rat cells may not be necessary for pseudotype production. Spontaneous fusion, believed to occur during co-cultivation (Migeon, 1968; Gabelman et al. 1975; Weiss & Lo-Wong, 1977) of the nonproducer cells and BEV infected cells, could result in rescue of MSV either by use of pre-existing BEV virus products, including the envelope glycoprotein, or by formation of heterokaryons in which transcription and translation of BEV genetic information could still occur. The fact that co-cultivation of HL23V producing cells with the same murine nonproducer cell lines failed to result in rescue of focus forming activity, although HL23V itself can replicate in these cells if introduced by means of the HL23V(BEV) pseudotype (data not shown), suggests that the fusion process thought to be required for sarcoma virus rescue to occur, may be, at least in part, mediated by virus glycoprotein produced by the rescuing infected cell.

The availability of a highly efficient selection system based on the observed properties of murine cells and of the viruses HL23V and BEV (Schnitzer et al. 1977), has permitted study of possible genetic interactions during the replication of these viruses in dually infected cells. In the absence of recombinants among the different species of possible virus released from permissive cells doubly-infected with HL23V and BEV, only the HL23V(BEV) pseudotype would be expected to result in productive infection of murine cells (see above). Infected murine cells would release only one type of virus particle, HL23V, but such virus would be unable to infect neighbouring murine cells in the original cell culture and hence would not be expected to spread with continued cell passage. However, if genetic recombination were to occur in cells doubly infected by HL23V and BEV with the production of a new virus now containing the genetic information for the host range for penetration of BEV and the replication ability of HL23V, such recombinants could spread to neighbouring,
uninfected murine cells with continued cell passage and would be expected to be capable of passage to further murine cell cultures. Despite repeated attempts to demonstrate the presence of recombinants in progeny from doubly infected cells, no reverse transcriptase activity was ever observed in supernatants from either the originally or secondarily infected murine cultures, even after more than ten passages of the originally infected NIH cells. These same supernatants were also examined for the presence of XC plaque-forming virus, using murine indicator cell monolayers in the XC assay to identify any recombinant virus having a glycoprotein which would now permit penetration of murine cells but still retain XC plaque-forming ability. Again, no recombinant virus of this type could be demonstrated.

In cells dually infected with different oncoviruses, virus interactions can occur at several levels and may be manifest by the appearance of recombinant virions (Weiss et al. 1973; Fischinger et al. 1975; Hayward & Hanafusa, 1975; Elder et al. 1977; Hartley et al. 1977), extension of host range of virus replication (Besmer & Baltimore, 1977; Ishimoto et al. 1977; Levy, 1977a, b; Weiss & Lo-Wong, 1977) and/or the production of phenotypically mixed particles (Gabelman et al. 1975; Zavada, 1976; Besmer & Baltimore, 1977; Ishimoto et al. 1977; Levy, 1977a; Weiss & Lo-Wong, 1977). Because of the observation that two oncoviruses, one identical to or closely related to BEV and the other to SSAV, have been infrequently but repeatedly isolated from human tissues in association with each other, our studies were undertaken to delineate what types of interactions could occur between these two oncoviruses.

Examination of progeny virus from permissive cells infected with both BEV and HL23V demonstrated that phenotypic mixing at the level of virus glycoprotein with the production of pseudotype particles was a regular and reproducible feature of their co-replication, similar to findings reported by other investigators involving interactions between both endogenous and exogenous oncoviruses as well as between two exogenous viruses, as in this case (Gabelman et al. 1975; Besmer & Baltimore, 1977; Ishimoto et al. 1977; Levy, 1977a, b; Weiss & Lo-Wong, 1977; Zavada, 1976). Whether phenotypic mixing also occurred at levels other than that of virus glycoproteins, as demonstrated by Hanafusa & Hanafusa (1968) in an avian virus system and suggested by Rein et al. (1976) in a murine virus system, was not investigated in these studies.

One consequence of the observed phenotypic mixing, the production of HL23V(BEV) pseudotype virus, permitted extension of the replicative host range of HL23V to murine cells. This observation, similar to those with oncovirus pseudotypes of MuLV-X (Besmer & Baltimore, 1977; Ishimoto et al. 1977; Levy, 1977a), was based on the ability of the HL23V
(BEV) pseudotype to overcome the block to HL23V penetration previously shown to be present in murine cells (Schnitzer et al. 1977) and provided evidence for the absence of any absolute post-penetration block to HL23V replication. The fact that the titre of HL23V released from infected NIH cells was always 100–1000 fold lower than that found from cells normally permissive for HL23V replication suggested, however, that in murine cells some mechanism preventing normal virus expression could still exist.

Another mechanism permitting extension of virus host range involves the ability of one virus to supply some replicative function lacking in a second co-infecting virus. BEV has been reported to be unable to replicate in rat or mouse cells (Todaro et al. 1976) yet penetrates these cells efficiently (Schnitzer et al. 1977). In experiments examining progeny virus from Rat-1 cells infected with virus from cells doubly-infected with HL23V and BEV, however, there was evidence for the expression in rat cells of BEV glycoprotein that was unlikely to have originated from the input virus, suggesting that integration and subsequent translation of some or all of the BEV genome had occurred. The possibility that BEV expression in Rat-1 cells was in some way a consequence of simultaneous infection by a second oncovirus (HL23V or, in the nonproducer, MSV) could not be ruled out. Direct infection of nonproducer cells with BEV never resulted in release of focus-forming activity suggesting that fusion between nonproducer cells and cells productively infected with BEV, as demonstrated by Weiss & Lo-Wong (1977) in a similar system, may be a necessary condition for such complementation to occur. That the fusion process may be at least partially mediated by virus glycoproteins was suggested by the fact that only BEV infected cells and not HL23V infected cells could rescue MSV from murine nonproducer cells, paralleling the host range of penetration rather than replication of the two viruses (Schnitzer et al. 1977).

The significance of these in vitro observations lies in their ability to suggest and support possible in vivo pathogenetic mechanisms. Evidence has been presented which demonstrates that the expression of BEV or BEV-related genetic information may occur in some human tissues (Sherr & Todaro, 1974; Strand & August, 1974; Rein et al. 1976; Wong-Staal et al. 1976); other biochemical data, as well as antibody studies, have suggested that HL23V infection in man may be a consequence of exposure to a naturally occurring infectious agent (Kurth et al. 1977; Teich & Weiss, 1977). The isolation of both these agents from human tumour tissue could result from the rare occasion of HL23V infection of a cell either expressing, or being induced to express BEV glycoprotein as a consequence of exogenous oncovirus infection (Fischinger & Nomura, 1975). It could then be hypothesized that the HL23V(BEV) pseudotypes thereby generated could infect certain cell types nonpermissive for HL23V replication because of a block to virus penetration, the expression of the virus genome in these cells then leading to malignant transformation and subsequent clinical disease. The inability of HL23V released from such transformed cells to infect other cells as a consequence of the block to HL23V penetration could explain the clonal nature of the malignancy.

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