Characterization of a Retrovirus Isolated from Normal Mink Cells Co-cultivated with a Dog Mammary Tumour

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SUMMARY

A retrovirus antigenically distinct from known type C, B and D viruses was isolated from normal mink (Mustela vison) lung cells that had been co-cultivated with 5-iododeoxyuridine- and dexamethasone-treated dog mammary tumour cells. Cytogenetic studies of the virus-releasing co-culture showed mitotic figures identical to the normal mink cell line (MvlLu) with the exception of a low frequency of cells with extensive chromosomal breakage and uncoiling. The new virus bands at a buoyant density of 1.16 g/ml, contains 60S RNA and a reverse transcriptase which prefers Mn2+ over Mg2+ for the synthesis of DNA. This enzyme utilizes poly(rA).oligo(dT) more efficiently than poly(dA).oligo(dT) and is also able to synthesize DNA copies from the endogenous RNA. Morphologically, it is a typical type C virus. Filtered virus readily infects mink, dog and other mammalian cells indicating the amphotropic nature of its cell growth requirement. Hybridization studies showed that normal mink DNA contains multiple copies of proviral sequences of this newly isolated virus. Serological analyses indicate that the mink endogenous virus contains in its core protein, in addition to the interspecies type-C determinant, an antigenic component related to one of the determinants found in the feline leukaemia virus p30 protein. This determinant is not present in the Rauscher leukaemia virus, RD114 virus or simian sarcoma virus.

Recently we co-cultivated dog mammary tumour cultures with several mammalian cell lines to search for virogenes in dog tumours. Among 19 different dog cultures that were co-cultivated with mink cells and studied, we found one co-culture that yielded fully mature infectious retrovirus. This report describes the physical, biochemical, immunological and genetic characterization of the new virus isolate.

Cell lines derived from normal mink lung (MvlLu) normal bat lung (TbILu), canine thymus (CfzTh), dog sarcoma (DI7), normal human leukocytes (NC37) and human rhabdomyosarcoma (A204) were used for co-cultivation with dog mammary tumour cells. Fresh dog mammary tumours were supplied by Dr A. Bogden of the Mason Research Institute, Worcester, Mass. U.S.A. Tumour cell clusters, after overnight treatment with collagenase, were diluted in either Dulbecco’s MEM containing insulin (10 µg/ml) and dexamethasone (10 µg/ml) or RPMI 1640 medium containing insulin (5 µg/ml), prolactin (0.5 µg/ml), oestradiol (0.05 µg/ml) and progesterone (0.05 µg/ml). All established cell lines were propagated in the RPMI 1640 medium; growth media contained 10 to 20% foetal calf serum.

Purified concentrated virus preparations (Pfizer Inc.) were obtained through the courtesy of Dr J. Gruber of the Office of Program Resources and Logistics, NCI, NIH, Md. U.S.A. Virus agents included Rauscher leukaemia virus (RLV), feline leukaemia virus (FeLV), cat endogenous RD114 virus, Mason–Pfizer monkey virus (M-PMV) and simian sarcoma virus (SSV). Monospecific antisera to p30 proteins of each of these viruses were produced.
either at the Maywood Pfizer laboratories or Huntingdon Research Center, Brooklandville, Md., U.S.A. Mouse mammary tumour virus (MMTV) was obtained from the Frederick Cancer Research Center, Frederick, Md., U.S.A. and the monospecific antisera to MMTV p28 and MMTV gp52 were kindly supplied by Dr N. H. Sarkar and Dr D. Fine.

Spontaneous mammary tumours from various breeds of dogs when cultured in growth media supplemented with hormones showed no cytopathic effects nor any other type of virus activity. Four weeks later attempts were made to isolate an endogenous virus by treating the cultures with 5-iododeoxyuridine (IdUrd, 25 μg/ml) for 48 h and then re-feeding the washed cells with growth medium containing dexamethasone (DXM, 10 μg/ml). After 3 days, the dog cultures were co-cultivated with several established cell lines. Of 19 dog cultures co-cultivated and tested for up to 20 weeks after co-cultivation, only one culture demonstrated the virus reverse transcriptase activity (RT). This culture was originated 18 weeks previously from basset dog mammary tumour cells that had been treated with IdUrd and DXM and then co-cultivated with mink cells. Neither the dog mammary culture nor the mink culture used for co-cultivation had any reverse transcriptase activity separately. Since the virus activity was observed in the co-cultivated culture, a series of biological, serological and biochemical tests were conducted to characterize and determine the origin of this new virus enzyme.

The species origin of the cells in the co-cultivated culture releasing the virus was compared cyogenetically with that of the normal mink lung culture used for the co-cultivation study (Ahmed et al. 1977). Cytogenetically, the normal mink and the co-cultivated cultures (releasing virus) were similar in that all mitotic figures observed were apparently of mink origin, and the modal chromosome number was 29 chromosomes. Representative karyotypes from either culture showed a normal male mink chromosome composition with the exception of a missing no. 9 chromosome. In addition to the hypodiploid but otherwise normal mink cells, the co-cultivated culture also showed a low frequency (less than 1%) of extensive chromosomal breakage and uncoiling.

The electron microscopic examination (Stephens et al. 1975) of the co-culture demonstrating RT activity revealed mature type C virus particles with dense central nucleoids (40 to 50 nm in diam.) surrounded by electron lucent envelopes (100 to 110 nm). First evidence of retroviral synthesis was realized on the plasma membrane in the form of crescent-shaped buds. Negatively stained virus preparations showed particles with head and tail morphology without surface spikes. In the beginning, some amorphous material was found to adhere to the virus envelope; later passages of the culture, however, did not show this phenomenon.

The radioactively labelled virus containing fractions from isopycnic sucrose gradients (buoyant density 1.16 g/ml) were pooled and the RNA was extracted (Michalides & Schlom, 1975). As determined by sedimentation velocity analysis in glycerol gradients, the virus RNA had a sedimentation coefficient of approx. 60S. To determine the species of origin of the virus, high mol. wt. 3H-virus RNA was hybridized to cellular DNAs of both mink and dog (Drohan et al. 1977b). The labelled virus RNA did not hybridize above background levels to the DNA of normal canine spleen indicating that the virus is not an endogenous canine virus (Table 1a). Furthermore, the virus failed to hybridize to DNAs from three canine mammary adenocarcinomas. Hybridization to feline DNA was also negative. 3H-virus RNA, however, hybridized extensively to DNA both from the established normal mink lung cell line (MvLu) and from pooled organs of a wild mink. More than 70% of the virus RNA hybridized to normal mink cellular DNA with a C.o of approx. 340 mol. s.1⁻¹. When polyadenosine selected RNA from normal murine cells was hybridized to murine
Table 1. Origin and host range of MiRV

A. Hybridization of $^3$H-virus RNA to cellular DNAs

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Percentage hybridized*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mink cell line (MvlLu)</td>
<td>73.5</td>
</tr>
<tr>
<td>Mink organs (pooled)</td>
<td>78.1</td>
</tr>
<tr>
<td>Canine spleen</td>
<td>7.3</td>
</tr>
<tr>
<td>Canine adenocarcinoma 1</td>
<td>2.0</td>
</tr>
<tr>
<td>Canine adenocarcinoma 2</td>
<td>4.0</td>
</tr>
<tr>
<td>Canine adenocarcinoma 3</td>
<td>3.3</td>
</tr>
<tr>
<td>Feline lung</td>
<td>6.5</td>
</tr>
<tr>
<td>E. coli</td>
<td>4.5</td>
</tr>
</tbody>
</table>

B. Host range studies with MiRV†

<table>
<thead>
<tr>
<th>Source</th>
<th>Cell line</th>
<th>$^3$H-TMP, ct/min/50 µl/h</th>
<th>IF‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine thymus</td>
<td>Cf2th</td>
<td>18463</td>
<td>+</td>
</tr>
<tr>
<td>Canine sarcoma</td>
<td>D17</td>
<td>18094</td>
<td>+</td>
</tr>
<tr>
<td>Canine mammary carcinoma</td>
<td>D14</td>
<td>4290</td>
<td>+</td>
</tr>
<tr>
<td>Mink lung</td>
<td>MvlLu</td>
<td>23164</td>
<td>+</td>
</tr>
<tr>
<td>Bat Lung</td>
<td>TblLu</td>
<td>87</td>
<td>−</td>
</tr>
<tr>
<td>Human rhabdomyosarcoma</td>
<td>A204</td>
<td>14394</td>
<td>+</td>
</tr>
<tr>
<td>Non-infection</td>
<td>Cf2th</td>
<td>593</td>
<td>−</td>
</tr>
</tbody>
</table>

* All hybridizations were carried out to a Cot of 15000.
† Cell lines were infected with cell-free preparations of MiRV and tested four weeks post-infection. Reverse transcriptase assays were conducted with clarified concentrated (100 x) culture fluid using poly(rA) as template. Immunofluorescence tests were carried out with a specific polyvalent antiserum prepared against disrupted MiRV.
‡ Immunofluorescence test.

DNA under similar conditions a Cot of approx. 3000 was obtained (Drohan et al. 1977b). Therefore, the kinetics of hybridization of virus RNA to normal mink DNA indicates that these sequences are present as provirus in the low repetitive range of cellular DNA sequences.

To determine the quality of hybrids formed between the $^3$H-virus RNA and normal mink DNA, the thermal stability of the hybrids was examined by elution from hydroxylapatite columns with 0.12M-sodium phosphate buffer in 5 °C increments from 60 to 100 °C. The dissociation of mink DNA was followed by measuring changes in absorbance at 260 nm and exhibited a melting point (Tm) of 87.5 °C. The dissociation of RNA-DNA hybrids, as followed by measuring acid precipitable radioactivity in eluent fractions from the hydroxylapatite column, exhibited a Tm of 84.5 °C. This latter Tm indicates that the virus RNA hybridized to totally complementary or extremely similar cellular DNA sequences (Drohan et al. 1977a). This is another indication that the virus is indeed of mink origin. The new virus isolate has been designated as MiRV.

The structural protein profile of MiRV, electrophoresed on polyacrylamide gels, was very similar to that of known type-C viruses. Serological experiments were performed to analyse the relationships of MiRV with RLV, SSV, M-PMV, RD114 and FeLV. In competition radioimmunoassays (Yeh et al. 1975), the MiRV preparations showed no competition with any of the viruses with the exception of FeLV (Fig. 1). A low level of competition, upwards
Fig. 1. Competition radioimmunoassays demonstrating relationships of MiRV with other mammalian retroviruses. MiRV was banded twice and disrupted with 0.05 M-tris buffer, pH 7.9, containing 0.25% Triton X-100 and 0.02 M-dithiothreitol and tested at various protein concentrations as competing antigen in homologous p30 assays of M-PMV, SSV, RD114 and FeLV. ◦–◦, Homologous p30; ◦––◦, cell extracts from cultures producing the homologous virus; ■–■, disrupted MiRV.

of 30% was observed with the FeLV system. The slope of this curve suggested some cross reaction between MiRV and FeLV p30. Immunodiffusion studies also showed that MiRV did not cross-react with p30 intraspecies determinants of RLV, SSV, M-PMV, MMTV and RD114. A precipitin line between MiRV and FeLV p30 antiserum which contained strong reactivities for intra- and interspecies antigens was observed. Analysis with this antiserum suggested that MiRV contains it in core protein, in addition to the interspecies type C determinant, an antigenic component found in FeLV p30. This determinant was not evident in RLV, SSV or RD114 virus.

The reverse transcriptase activity of MiRV was characterized with regard to the divalent cation requirement. Various concentrations of MnCl₂ or MgCl₂ were added to the DNA polymerization mixture for optimal activity and the incorporation of ³H-TMP into a synthetic template primer poly(rA).oligo(dT) was compared to that using poly(dA).oligo(dT) (Kimball et al. 1976). Maximal incorporation was observed at a Mn²⁺ concentration of 0.2 mM. Higher Mn²⁺ concentration was inhibitory. The optimal Mg²⁺ concentration was found to be 5.0 mM; however, Mg²⁺ was only 12% as effective as Mn²⁺.

Cell-free filtered or density gradient purified preparations of MiRV were added to several different established cell lines. The cells were subcultured weekly and the culture supernatants were analysed for RT activity at four weeks post infection. As shown in Table 1B the RT enzyme was detected in virus inoculated mink, canine and human cell lines but not in the
bat lung culture. Immunofluorescence studies using a specific antiserum prepared against MiRV showed cytoplasmic fluorescence only in the RT-positive cultures.

The interaction between MiRV infected mink cells and XC or KC cells carrying the RSV genome was evaluated. Infected mink cells were streaked or irradiated for 30 s with u.v. light (60 erg/mm²) and XC or KC cells (1 to 2 × 10⁶ cells) were added (Ahmed et al. 1974). No syncytium formation was observed with either types of RSV transformed cells following incubation up to 96 h at 37 °C.

Since MvlLu cells are susceptible to infection by a variety of retroviruses, they have been extensively used for isolation and propagation of xenotropic mouse and primate endogenous retroviruses. This is also one of few cell lines that can support the replication of mouse mammary tumour virus (Lasfargues et al. 1977). Furthermore, the culture allows the development of foci of transformed cells following inoculation with murine and feline sarcoma viruses (Henderson et al. 1974; Peebles, 1975). The role of endogenous retrovirus sequences in replicative cycles of exogenously infected viruses remains to be determined.

It is interesting to note that MvlLu cells did not transcribe retrovirus sequences into complete virions unless dog tumour cells were co-cultivated with them. Mink cells were maintained, with weekly subculturing, for more than two years without the evidence of spontaneous release of a retrovirus. Several experiments designed to activate the virogene with IdUrd and DXM also failed, although MiRV structural proteins could be detected in the mink cells, with or without chemical treatment, by immunofluorescence tests. This suggests that co-culturing of mink cells with dog tumour or other cell lines may provide the necessary information needed for active multiplication of endogenous type C virus in mink cells.

Following isolation of the endogenous mink virus described here (MiRV) we learned that type C viruses were isolated from mixed cultures of baboon cells and Kirsten sarcoma virus transformed mink cells (Scherr et al. 1978), normal mink cells co-cultivated with 5-BrdUrd treated mouse cells (Klement et al. 1978) and directly from a mink cell line (Barbacid et al. 1978). Nucleic acid hybridization experiments indicated that these viruses are endogenous to mink. Host range studies by us and others show that there exist some differences among various mink retrovirus isolates. While the mink virus reported here appears to be amphotropic in nature, the virus isolated by Scherr et al. (1978) is xenotropic and has a very limited host range. The virus isolate of Klement et al. (1978) has a wider host range and although it replicates in mink cells, the growth is minimal. MiRV, on the other hand, readily infects mink cells and is able to complete its replicative cycle intracellularly (MiRV replication and maturation in mink cells were confirmed by reverse transcriptase assays, immunoassays and electron microscopy). In view of the great heterogeneity among type C retroviruses isolated from a given species, such as in the case of the mouse and the cat, it will be of interest to compare the MiRV isolate described here with the present and future retrovirus isolates from mink cells.

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REMARKS


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