Detection of Simian Type-C Particles in Mason-Pfizer Virus Infected Cultures

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SUMMARY

A simian type-C virus has been detected in cultures chronically infected with Mason-Pfizer monkey virus (M-PMV). Simultaneous budding of M-PMV and type-C virus particles from the same cells was observed in cultures incubated at 37 or 40 °C. However, the frequency of such cells was greater in cultures grown at 40° C. Although clusters of type-C viral buds were seen at the surface of the cells, extracellular mature type-C particles in cell pellets or concentrated virus preparations were very rarely found. The increase in frequency of type-C buds was found to be transitory since cultures adapted to growing at the high temperature demonstrated budding type-C particles only occasionally. Cultures producing type-C buds were found to contain, in addition to M-PMV antigens, serological activity with polyvalent antisera produced against multiple structural components of endogenous baboon virus (BV) or simian sarcoma virus (SiSV). The reactivity, however, was found not to be serologically related to the major SiSV p28 core protein.

INTRODUCTION

Mason-Pfizer monkey virus (M-PMV) was isolated from a spontaneous mammary adenocarcinoma of a female rhesus monkey (Chopra & Mason, 1970; Mason et al. 1972). The infected cells contain intracytoplasmic particles that are enveloped during budding as is the case with type-B particles of the mouse mammary tumour virus; the virus envelope, however, is smooth and does not have any spikes (Kramarsky, Sarkar & Moore, 1971; Manning & Hackett, 1972; Ahmed et al. 1974). Several attempts to culture the rhesus mammary tumour by itself failed. However, when the tumour was co-cultivated with normal monkey foetal cells, the culture grew and a cell line was established (Jensen et al. 1970). The co-cultivated monkey mammary tumour (CMMT) culture was shown to contain virus morphologically identical to the particles seen in the original mammary tumour biopsy. The virus from CMMT cultures was successfully used to establish a chronic infection of a normal human lymphoblastoid suspension cell line (Jensen et al. 1970; Chopra et al. 1971).

This paper describes the finding of type-C particles in the original CMMT cell line as well as in other cells infected with M-PMV. The virus develops and buds like the type-C virus particles of mammalian species. The cultures, when incubated at 40 °C, show a transitory increase in the amount of budding type-C virus particles. In addition, a type-C viral antigen has been detected in these cultures when reacted with polyvalent antisera prepared against endogenous baboon virus (BV) and simian sarcoma virus (SiSV).
METHODS

Cell lines. Cell lines chronically infected with M-PMV (CMMT and human lymphoblastoid cultures), Rauscher leukaemia virus (RLV; JSVL-9 culture), feline leukaemia virus (FeLV; FL-74 culture), rat type-C virus (R-35 mammary tumour culture), simian sarcoma virus (SiSV; human lymphoblastoid culture) and endogenous baboon virus (BV; human lymphoblastoid culture) were propagated in RPMI no. 1640 medium with 10 to 15% inactivated foetal calf serum. All cell lines were passaged once every 6 to 10 days and incubated at 37 °C unless otherwise stated. M-PMV infected cultures were propagated in a separate laboratory.

Antisera. Polyvalent antisera to M-PMV, SiSV, BV, RLV, rat type-C virus and FeLV sera were produced in rabbits. The immunization was carried out with sucrose density gradient purified virus preparations containing $1 \times 10^{11}$ to $1 \times 10^{18}$ virus particles/ml which had been disrupted by freeze-thawing (six times) and sonication (Branson Sonic Power Co., Plainview, Long Island, N.Y., model WI40D, at an output intensity of 50% for 3 min) before inoculation. Polyacrylamide gel electrophoretic (PAGE) analysis of unlabelled immunizing viral antigens demonstrated at least 10 different viral structural proteins and glycoproteins. Similarly, monitoring by PAGE of several viruses labelled either with [3H]-amino acids during viral synthesis or with $^{125}$I after virus purification and disruption, showed more than 8 protein peaks for each virus. Polyvalent antisera prepared against multiple viral structural components produced 4 to 6 precipitin lines with homologous immunizing viral antigens demonstrated at least 10 different viral structural proteins and glycoproteins. Similarly, monitoring by PAGE of several viruses labelled either with [3H]-amino acids during viral synthesis or with $^{125}$I after virus purification and disruption, showed more than 8 protein peaks for each virus. Polyvalent antisera prepared against multiple viral structural components produced 4 to 6 precipitin lines with homologous disrupted virus in the microimmunodiffusion tests (Ahmed et al. 1970). Mouse mammary tumour virus (MuMTV) antiserum was kindly supplied by Dr J. Charney & Dr D. Moore, Institute for Medical Research, Camden, N.J. A polyvalent antiserum to SiSV was received through the courtesy of Dr L. Wolfe, Rush-Presbyterian-St Luke’s Medical Center, Chicago, Ill. It was prepared in a rabbit using Tween-ether-treated SiSV. Monospecific antiserum to purified major protein of SiSV, mol. wt. 28,000 (SiSV p28), prepared in a goat was a gift from Dr J. T. August, Albert Einstein College of Medicine, New York. Monospecific antiserum to purified SiSV p28 was also produced in rabbits in our laboratory. The two monospecific antisera showed an identical reactivity and specificity in the immunofluorescence and immunodiffusion tests. Anti-Moloney sarcoma serum (MSS) produced in rats was obtained through the courtesy of Dr R. Wilsnack, Huntington Research Labs, Baltimore. It contained intra- and interspecies-specific antibodies.

Serological tests. Microimmunodiffusion (MID) tests and indirect immunofluorescence (IF) tests were performed as reported previously (Ahmed et al. 1971).

Electron microscopy. Cell pellets were fixed for 2 h at room temperature in 2% glutaraldehyde (Ladd Research Inc.) in 0.05 M-phosphate buffered saline (PBS), and post-fixed for 2 h in Chrome-Osmium (Dalton, 1955). After a thorough rinse in water the pellets were kept overnight in 2% uranyl acetate in 50% ethanol, then rapidly dehydrated in increasing concentrations of ethanol and embedded in Epon according to Luft (1961). Sections were stained with lead citrate (Venable & Coggeshall, 1965).

RESULTS

Effect of incubation temperature

In the course of periodic electron microscopic examination of CMMT and the infected human lymphoblastoid cell lines propagated at 37 °C, it was noticed that a few cells were exhibiting type-C buds along with M-PMV buds and cytoplasmic type-A particles. How-
ever, such cells were very rare and the detection of type-C particles proved to be highly inconsistent. The CMMT cell line was subcultured and incubated in parallel at 33 °C (CMMT-33), 37 °C (CMMT-37) and 40 °C (CMMT-40). The patterns of cell growth of CMMT-33 and CMMT-40 were markedly different from those of CMMT-37. CMMT-33 cultures grew very slowly and showed some multinucleated cells after a week of incubation. On the other hand CMMT-40 cultures demonstrated toxicity and only 20 to 10% of cells were seen attached to the flask following a week of incubation (Fig. 1, 2). The growth medium of the high-temperature experiments was frequently changed to get rid of the floating debris. Initial heat trauma appeared to decrease in cultures that survived for 3 to 4 weeks and further propagation resulted in complete adaptation of these cultures to 40 °C. The cultures grown at various temperatures were examined for virus multiplication and viral antigens.

Electron microscopy

Major features of typical M-PMV particle production in a chronically infected human lymphoblastoid cell line grown in 37 °C are illustrated in Fig. 3. Type-A particles (at A arrows) appear as circles with a peripheral area denser than the central core and are described as doughnuts (insert a). They are usually found in clusters and always directly within the cytoplasmic matrix. Within the clusters are frequently found numerous profiles of membranous vesicles which are never seen to envelop the type-A particles. The clusters are usually somewhat removed from the cell surface. The budding M-PMV particle (at M arrow)
Fig. 3. M-PMV in a human lymphoblastoid cell grown at 37 °C. Cytoplasmic type-A particles at A arrows. M-PMV buds at M arrows on cell surface. Compare the appearance of nucleoids in budding particles with that of type-A particles in the cytoplasm. (a) Intra-cytoplasmic type-A particle. Note the fringes at the periphery of the doughnut.

Fig. 4. Same culture as in Fig. 3. Numerous viral buds are present at the periphery of this mitotic cell. A cluster of the type-A particles is outlined by the large circle. The viral buds outlined by small circles are magnified in Fig. 5 and 6.

Fig. 5. Enlargement of type-C viral bud appearing within lower small circle in Fig. 4. The nucleoid consists of a crescent of denser material (C) which is separated from the viral envelope by a space of lower density.

Fig. 6. Same viral bud as within upper small circle of Fig. 4. The nucleoid (M) is doughnut-shaped and typical of M-PMV-type buds.
Fig. 7. Several viral buds on the surface of a CMMT cell incubated at 40 °C. Type-C buds with crescent and cylindrical nucleoids at white arrows. M-PMV-type buds with doughnut-nucleoids at black arrows.

Fig. 8. Portion of cell with convoluted surface exhibiting numerous viral buds in the CMMT culture grown at 40 °C. A cylindrical type-C bud is shown within the large circle and extracellular mature M-PMV particle within the small circle. (a) Further enlargement of cylindrical type-C particle. (b) Extracellular mature M-PMV. Note the typical M-PMV nucleoid which at this stage has condensed from a doughnut-shape to an elongated shape surrounded by an intermediate line.
consists of a doughnut-shaped centrally located nucleoid which becomes enveloped by a portion of the plasma membrane. Such nucleoids bear a striking resemblance to the cytoplasmic type-A particles and are always seen as complete doughnuts throughout the whole budding process.

Cells simultaneously exhibiting viral buds with doughnut- or crescent-shaped nucleoids were observed in infected simian and human cultures propagated at 37 °C (Fig. 4 to 6) as well as when incubated at 40 °C for one week (Fig. 7, 8 and 10). However, the frequency of such cells was greater in cultures incubated at 40 °C. In cultures grown at 33 °C, most of infected cells contained only type-A particles. A few viral buds with doughnut-shaped nucleoids were also seen but buds with crescent-shaped nucleoids were not observed. Fig. 5 and 6 compare the appearance of the two types of viral buds found on the plasma membrane of an infected human cell illustrated in Fig. 4. The viral bud with a crescent-shaped nucleoid (Fig. 5) is identical to the viral buds seen in type-C virus, while the bud with a doughnut nucleoid (Fig. 6) is that of M-PMV.

Type-C buds were also seen along with regular M-PMV buds in the CMMT-40 culture (Fig. 7 and 8). Inserts a and b in Fig. 8 represent high magnification of a cylindrical type-C bud and an extracellular mature M-PMV particle respectively. The nucleoid in the mature M-PMV particle is elongated and consists of a central dense portion surrounded by an intermediate line. This typical appearance of mature M-PMV particles is also illustrated in Fig. 9 at higher magnification. Cells budding both M-PMV and type-C particles were frequently found to have a highly convoluted surface in cultures incubated at 40 °C. The presence of a cluster of type-C buds on such a culture is shown in Fig. 10. Although these clusters of type-C buds were seen at the surface of CMMT cells incubated at 40 °C, extracellular type-C particles in cell pellets or concentrated virus preparations were very rare. Moreover, the increase in frequency of type-C buds in cultures grown at 40 °C was transitory since cells with type-C buds were seen only occasionally after the cultures had been adapted to growing at high temperature.

The type-C buds in M-PMV infected cultures were identical to those described for endogenous type-C virus in placentas of baboons (Kalter et al. 1973) and rhesus monkeys (Schidlovsky & Amed, 1973).

Serological studies

In order to identify different antigens in CMMT cultures, acetone fixed preparations of cells were tested by immunofluorescence with polyvalent antisera to RLV, MuMTV, rat type-C virus, FeLV, SiSV, BV and M-PMV. As shown in Table 1, M-PMV antigen was demonstrable in 60 to 85% of cells propagated at various temperatures. The CMMT-33, CMMT-37 and CMMT-40 also contained antigens that reacted with polyvalent SiSV and BV antisera, though fluorescence was evident in fewer cells from cultures kept at 33 and 37 °C than from those incubated at 40 °C. Moreover, CMMT-40 cells elicited a much more intense and brilliant fluorescence with the positive antisera than did those incubated at lower temperatures. The immunofluorescence attributed to M-PMV, BV or SiSV proteins was demonstrable only in the cytoplasm of infected cells. While polyvalent simian type-C viral antisera reacted with CMMT cultures, the monospecific antiserum to SiSV p28 did not, indicating that the cross reacting antigen in CMMT cells is not related to the major SiSV p28 protein. Antisera directed against other viruses (RLV, MuMTV, FeLV, rat type-C virus) failed to react with CMMT cells. Complement fixation (CF) tests showed that whereas CMMT-33, CMMT-37 and CMMT-40 cell extracts reacted with polyvalent SiSV antiserum, the titres were lower with CMMT-33 (1:32), and CMMT-37 (1:32) than with CMMT-40 (1:128).
In order to determine the presence of mammalian type-C virus interspecies-specific antigen, CMMT-cell extracts with appropriate controls were tested against a Moloney sarcoma serum (MSS) that had been shown to contain interspecies-specific (in addition to group-specific) antibodies by MID technique. No precipitin line appeared. The MSS also did not react with CMMT-40 cells in either IF or CF tests.
Table 2. Percent immunofluorescent-positive cells in CMMT cultures incubated at various temperatures

<table>
<thead>
<tr>
<th>Cell incubation temperature (°C)</th>
<th>Polyvalent antiserum against:</th>
<th>Monospecific antiserum against:</th>
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<tr>
<td></td>
<td>M-PMV</td>
<td>SiSV</td>
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<tr>
<td>33</td>
<td>60</td>
<td>10</td>
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<tr>
<td>37</td>
<td>82</td>
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<td>40</td>
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* Acetone-fixed cell preparations were stained with appropriate polyvalent or monospecific antiserum by the indirect immunofluorescence technique.

DISCUSSION

The virus originally described in M-PMV infected cultures consisted of four morphological entities: (1) doughnut-shaped cytoplasmic type-A particles; (2) virus buds at the plasma membrane containing doughnut-shaped nucleoids; (3) immature or primary extracellular particles consisting of an enveloped doughnut nucleoid, and (4) a mature or secondary extracellular particle consisting of an enveloped cylindrical nucleoid surrounded by an intermediate membrane. The type-A particles resembled those seen with type-B virus found in mouse mammary tumours. The M-PMV buds also resembled those seen in MuMTV infected cultures in that their nucleoid was always doughnut-shaped but differed from them in that their envelopes did not have any surface spikes. (Jensen et al. 1970; Kramarsky et al. 1971; Manning & Hackett, 1972; Ahmed et al. 1974).

In the present study both CMMT and infected human cell lines yielded, in addition to M-PMV, a budding type-C virus. The incubation of cultures at 40 °C showed a definite, although transient, increase in production of type-C buds. Several aberrant cylindrical forms with an electron dense core were also seen. Although the fate of these structures in the extracellular space is unknown, they have been described in type-C virus in vivo (Dalton, Haguenau & Moloney, 1964; Feldman & Gross, 1967) and in vitro (Hall, Anderson & Evans, 1967). Clusters of type-C viral buds were seen in CMMT cultures incubated at 40 °C, but extracellular mature type-C particles were observed only very rarely. This may indicate some inhibition in the release or maturation of type-C virus at high temperature. Moreover, the induction of type-C buds in these cultures was transitory. Cultures that had been adapted to growing at 40 °C were frequently free of type-C buds. The cell morphology and cell growth patterns in high temperature adapted cultures were found to be the same as in cultures propagated at 37 °C.

Rhesus CMMT cells producing type-C buds were shown to contain, in addition to M-PMV antigens, a simian type-C viral antigen that showed cross reactivity with endogenous BV and SiSV. Since polyvalent antisera prepared against multiple antigens of BV or SiSV were employed in this study, the nature of the cross reacting antigen(s) could not be determined. However, it is important to note that the cross reactivity is not due to the major SiSV p28 antigen, because the monospecific antiserum prepared against this protein failed to react with CMMT cultures or purified M-PMV preparations. Mayer, Smith & Gallo (1974) have shown recently that the reverse transcriptase of BV and rhesus placental type-C virus are serologically related. These enzymes, however, are not inhibited by antibodies to the reverse transcriptases found in primate type-C viruses with known oncogenicity (Mayer et al. 1974; Sherr et al. 1974).

Efforts to demonstrate mammalian type-C interspecies-specific antigen in CMMT-40
Type-C particles in M-PMV infected cultures

culture by MID, IF or CF were unsuccessful. It appears that the M-PMV infected culture either lacks the interspecies component or contains it in amounts considerably less than cultures infected with FeLV, MuLV or rat type-C virus, since preparations from the latter cells readily demonstrated the interspecies antigen under the conditions used.

The serological data indicate that the type-C virus in CMMT cultures is a simian virus, and therefore, we designate it as simian type-C virus (SCV). There could not have been an inadvertent contamination of cultures with SiSV, gibbon ape lymphoma virus (GALV) or BV, since SCV was observed in CMMT cultures before these simian viruses arrived at the John L. Smith Memorial Laboratories. Furthermore, the CMMT cells frozen at passage 17, before the discovery of SiSV, GALV and BV, have been found to contain SCV antigen.

Two explanations for the appearance of SCV in the CMMT cell line can be advanced. The first is that SCV or its genome was present in the original monkey mammary tumour along with M-PMV. Culture conditions, especially incubation at high temperature, accentuated the production of SCV. In the mouse mammary tumour system, type-C particles have been observed along with type-B particles in the milk, mammary tumours and cell cultures originating from spontaneous and induced mammary tumours of inbred mice (Dmochowski et al. 1967, 1968; Calafat, 1968; Lasfargues et al. 1970a). Lasfargues et al. (1970b) observed that cultures of mouse mammary glands, after infecting with inocula containing type-B virus particles, produced type-C virus particles. Hilgers et al. (1971) reported simultaneous detection of type-B and type-C particles in the D.W. cell line derived from a spontaneous mouse mammary tumour. The immunofluorescence and mixed haemadsorption studies demonstrated type-B and type-C virus-specific antigens in this cell line. Simultaneous detection of type-B and C particles in the normal breast tissue, milk and mammary carcinoma of wild mice has also been reported (Rongey et al. 1973).

A second explanation is that the SCV or its genome was present in the normal rhesus monkey foetus used for original co-cultivation with the mammary tumour. Although no virus of any kind was seen by electron microscopy in the normal monkey foetal culture, the cells could have been carrying the SCV genome which was activated during co-cultivation. Recent findings of endogenous type-C virus in normal baboon and rhesus monkey placentas (Kalter et al. 1973; Schidlovsky & Ahmed, 1973) as well as in the rhesus umbilical cord and foetal tissues (Schidlovsky & Ahmed, 1973) supports this possibility. Therefore the type-C virus may have entered this culture system simultaneously by both routes, i.e. the tumour tissue and the embryonic tissue.

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