New Type B Retrovirus Isolates Associated with Kinetochores and Centrioles of the Host Cell

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SUMMARY

Two new virus isolates, M432 and M832, obtained from the Southeast Asian mouse have been characterized morphologically with respect to their composition and intracellular assembly. The mature virions resemble in certain respects the type B murine retroviruses. The new isolates, however, have an intracellular precursor, a type A particle, closely associated with the mitotic apparatus. The intracellular transport of the type A particles to the cell surface, where they are released by budding, is closely associated with the microtubule system of the cell.

INTRODUCTION

Two new retroviruses have been isolated recently from two different species of the Southeast Asian mouse. One virus, designated M432, was obtained from Mus cervicolor (Callahan et al. 1976), another virus, M832, from Mus caroli. Both have been described with respect to their immunological and biochemical behaviour (Callahan et al. 1978). The two isolates share certain characteristics and show some similarities to the murine mammary tumour virus, a retrovirus of type B, yet they can readily be differentiated both from each other and from known retroviruses of the mouse, both of type B and C, by their RNA genome and protein composition, as well as by their reverse transcriptase activity. On the basis of the data obtained, it was proposed that these virus isolates represent members of a new class of genetically transmitted murine retroviruses.

In this communication we wish to report on the ultrastructural composition of these virus isolates and on the manner of their intracellular assembly. These viruses can be differentiated morphologically from each other by the structure of their virus envelopes, both in ultrathin sections and in negatively stained virus preparations. The maturation of the virion involves an intracytoplasmic virus precursor resembling the intracytoplasmic particle of type A (Bernhard, 1973). The intracytoplasmic type A precursor particles are associated mainly with the mitotic apparatus. It is suggested that these observations may be of more general significance in understanding the manner of intracellular assembly of the type B Retroviridae.

METHODS

Viruses and cell lines. The isolation of the viruses and their propagation in different cell lines has been described previously in detail (Callahan et al. 1978). Briefly, the M432 virus, isolated from spleen cells of M. cervicolor (Callahan et al. 1976), was grown in NIH/3T3 cells (Jainchill et al. 1969) and the M832 virus, released spontaneously from M. caroli cells
Fig. 1. (a to c) Virus of strain M432; spikes are well recognizable on the surface of the virions (arrows). (d to f) Virus of strain M832; spikes are not clearly demonstrable. (a), (d) Ultrathin section of glutaraldehyde fixed material, postfixed with chrome-osmium and embedded in Epon-Araldite. (b), (e), (e) and (f), Negatively stained virus preparations. Magnification approx. 150,000.

(Lieber et al. 1973), was transmitted to and grown in Mus musculus SC-1 cells (Hartley & Rowe, 1976). The infected, virus-releasing cells were maintained in Dulbecco's MEM medium supplemented with 10% inactivated foetal calf serum, 40 units/ml penicillin and 40 µg/ml Fungizone.

Cells of different strains known to be either virus free (strains SC-1 and NIH/3T3) or harbouring different kinds of retroviruses [strain NIH/3T3 infected with Rauscher leukaemia virus of type C, strain Mm5mt/el (mouse mammary tumour virus of type B), strain Ki MSV transformed BALB 3T3 cells (intracisternal type A particles) and strain CHO, known to contain virus of type C as well as intracytoplasmic type A particles] served as controls.

Colcemid inhibition. Cells were arrested in metaphase by the addition of 0.05 µg/ml or 0.1 µg/ml Colcemid (Grand Island Biological Co., Grand Island, N.Y.) to the medium according to Stubblefield & Klevecz (1965). Cultures were treated with Colcemid for a period of 2 to 24 h and then either fixed immediately for electron microscopic observation or resuspended in fresh medium and fixed at different times after Colcemid removal.

Electron microscopy. All procedures were carried out at room temperature to obtain optimal preservation of microtubules. Cells were fixed as described previously (Heine, 1969), stained en bloc with 2% uranyl acetate in 50% alcohol, dehydrated in a series of alcohol
Fig. 2. Cell infected with virus of strain M432. A cluster of type A particles can be seen adjacent to centriole (C) and microtubules (M). The fibrillar matrix (F) is distinct from the surrounding cytoplasm containing numerous ribosomes. Two incomplete crescent-shaped particles are illustrated (long arrows). Fibrils attached to particles are clearly recognizable (short arrows). Magnification approx. 110000.
Fig. 3. Development stages of the virus. (a) Virus of strain M832; crescent-shaped particle with a thick, inner shell of about 25 nm and a thin, outer shell of about 10 nm diam. (b to f) Virus of strain M432. Intracytoplasmic (b), budding (c, d, e) and immature extracellular virions (f) are shown. Fibrillar and microtubular structures attached to the virion are clearly recognizable in (b). Magnification approx. 150000.

and embedded in Epon-Araldite. Sections of silver interference were double stained conventionally with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop 1A.
In addition, virus, suspended in distilled water, was deposited on carbon-coated grids and negatively stained with 0.5% potassium phosphotungstate (pH 6.8).

RESULTS

The mature virions

The mature virions of both strains exhibit an electron dense, centrally situated nucleoid surrounded by an intermediate shell and an enveloping membrane derived from the plasma membrane (Fig. 1a, d). As shown in Table 1, the size of the virions ranges between 100 and 120 nm in diam. The diam. of their nucleoids average about 90 nm. The virions of strain M432 are clearly distinguishable from those of strain M832 in ultrathin sections and in negatively stained preparations of virus obtained from tissue culture supernatants. As illustrated in Fig. 1(a, b and c), virions of strain M432 exhibit knob-like projections arranged in highly ordered fashion on their surface. Although these projections are recognizable in ultrathin sections (Fig. 1a), they can be especially well resolved in negatively stained preparations (Fig. 1b and c). The length of each protrusion is about 80 to 90 Å, their width is about
Table 1. Size of intracytoplasmic type A particles and extracellular virions

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>Intracytoplasmic type A particles</th>
<th>Extracellular virions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Centriole associated (nm)</td>
<td>Cytoplasmic (nm)</td>
</tr>
<tr>
<td>M432</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>M832</td>
<td>90</td>
<td>.</td>
</tr>
<tr>
<td>Mm5mt/cl</td>
<td>90</td>
<td>75</td>
</tr>
</tbody>
</table>

* Immature virions are those containing an electron-dense shell surrounding an electron-lucent centre, mature virions have an electron-dense nucleoid.

Fig. 4. Intracytoplasmic type A particles in cells infected with virus of strain M432. Particles are near the cell membrane. Apposition of microtubules is especially evident (arrows) in (b) and the fibrillar coat surrounding the particles is recognizable in (a) (arrowhead). Magnification approx. 120000.

60 Å, and each one has a hollow centre. In contrast, M832 virus does not possess surface protrusions of such a regular pattern (Fig. 1d, e, f). In the latter case, surface projections are not visible in ultrathin sections and negatively stained preparations reveal only an irregular, fuzzy coat on the virus surface. Mature virions of both strains are found in extracellular spaces; but are infrequently seen also in the cytoplasm where they accumulate within vacuoles and lysosomes of different size.

The distribution of intracellular virus precursors in interphase cells

In both strains studied, the appearance of the intracellular virus precursors and their relationship to the host cell are quite similar; therefore, the data referring to them will be discussed together.

In interphase cells, the intracytoplasmic precursor particles are relatively rare and when
Fig. 5. Cell infected with virus M432 arrested in metaphase in the presence of Colcemid. Accumulation of intracytoplasmic type A particles at the kinetochore plates (arrows) is shown. C = chromosomes. Magnification approx. 16000.

present are observed in clusters in close association with centrioles – only occasional single particles are observed at other sites of the cytoplasm (Fig. 2). As shown in Fig. 2 and 3, these particles represent virus precursors. They resemble, only in certain aspects, type A particles which are known to be present in large numbers in the cytoplasm of cells infected with the murine mammary tumour agent or the type B virus of the guinea pig. Four different components are recognizable in these particles. The most striking is an electron-dense, doughnut-shaped core with an outer diam. of 90 nm (Table 1), consisting of a broad inner shell of about 25 nm in thickness and a thin outer shell of 10 nm thickness (Fig. 3a). However, in contrast to the type A particles of the mammary tumour agent, the electron
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Fig. 6. Cell infected with virus M432 arrested in metaphase. Type A particles near the kinetochore (K) are illustrated in (a), and the crescent-shaped precursor (arrow) of the type A particle is near the chromosome (C) in (b). Magnification approx. 80000.

density of the outer and inner shell is equivalent in the particles studied here. Both shells enclose the electron-lucent centre of the particle. The surface of each virus particle is surrounded by a regular, halo-like and fibrillar coat of low electron density (Fig. 2).

When adjacent to the centriole, the particles are embedded in the centrosomal area, a fibrillar matrix, free of ribosomes, which can easily be distinguished from the surrounding cytoplasm (Fig. 2). In rare instances, incomplete precursor-like type A particles have been seen in this fibrillar matrix near the centrioles (Fig. 2). These incomplete particles are crescent-shaped structures consisting of a distinct inner and outer shell (Fig. 3a). They are comparable in size to the doughnut-shaped intracytoplasmic particles of type A.

As shown in Fig. 3(b to e), the doughnut-shaped type A particle progresses through the cytoplasm (Fig. 3b, c) to the plasma membrane and is released into the extracellular space by budding (Fig. 3d, e).

Close association of type A virus particles with microtubules is frequently observed both in the vicinity of centrioles and, when found, in other areas of the cytoplasm including the vicinity of the cell membrane (Fig. 3b, 4a and b).

The distribution of intracellular virus precursors during the mitotic cycle

The frequent close association of type A particles with microtubules and centrioles led us to study the distribution of these particles within the mitotic apparatus, where both centrioles and microtubules are incorporated into the mitotic spindle.

After exposure to Colcemid (see Methods), about 60 to 70% of the cells infected either with isolate M432 or M832 are found in metaphase arrest. Remarkably, type A particles can be found in the vicinity of the kinetochores of several chromosomes in all of these cells (Fig. 5 and 6). This phenomenon has been observed with regularity in over 500 cells.
Fig. 7. Cell infected with virus of strain M438 one hour after removal of Colcemid. Chromosomes are in late metaphase. Type A particles are no longer in contact with the kinetochores but are accumulating at microtubules near the centriole. Magnification approx. 16000.
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Fig. 8. Enlargement of Fig. 7 shows the close contact of type A particles with microtubules (arrows) near centriole (C). Magnification approx. 80,000.

arrested in metaphase. Preliminary results from examination of serial sections would indicate that this phenomenon is not confined to a few chromosomes but was observed generally at kinetochore regions of many chromosomes, and virus precursors could not be detected in other areas of the cells. Both complete, doughnut-shaped particles as well as their precursor, the crescent-shaped forms, are abundant in proximity to the kinetochore plate where they are always situated at the distal side of the kinetochores (Fig. 6a).

After removal of the drug, the cells recover rapidly and complete the mitotic cycle in about one hour. During this time the type A virus precursors lose their intimate association with the kinetochores yet retain their close association with microtubules as illustrated in Fig. 7 and 8, both representing a section through a cell during late metaphase fixed 60 min after drug removal.

In Table 2 we have summarized the findings in respect to the presence of intracytoplasmic
Table 2. The presence of intracytoplasmic type A particles at centrioles and kinetochores in different cell strains

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type of virus found</th>
<th>Number of cultures exposed to Colcemid</th>
<th>Colcemid concentration</th>
<th>Accumulation of type A particles at kinetochores and centrioles</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH/3T3</td>
<td></td>
<td>2</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>NIH/3T3 infected with M432 virus (extracellular)</td>
<td>B</td>
<td>4</td>
<td>0.05</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>NIH/3T3 infected with Rauscher virus</td>
<td>C</td>
<td>1</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>SC-1</td>
<td></td>
<td>4</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>SC-1 infected with M832 virus (extracellular)</td>
<td>B</td>
<td>3</td>
<td>0.1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>NIH/3T3 infected with Mm5mt/cl virus</td>
<td>B</td>
<td>1</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>KI MSV transformed BALB 3T3 cells</td>
<td>A</td>
<td>5</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>CHO</td>
<td></td>
<td>3</td>
<td>0.1</td>
<td>+</td>
</tr>
</tbody>
</table>

* Cells were exposed to Colcemid for various times ranging between 2 and 24 h.

Type A particles at kinetochores and/or centrioles in cells of different origin. To date, there are four cell strains known that harbour these particles at the specified locations. They are the previously described strain CHO (Wheatley, 1974; Gould & Borisy, 1976, 1977), strain PTK 1 (R. Brinkley, personal communication), and strains NIH/3T3 and SC-1 infected with virus M432 and M832, respectively, as reported in this study. In addition to the cells studied here, we have observed the accumulation of type A particles at the kinetochores of CHO cells arrested in metaphase after addition of Colcemid. Thus far, in spite of a careful and extensive search, we did not detect the same relationship between the type A particles and cells in five other cell strains, including controls known to be free of detectable extracellular virus and strains that carry other retroviruses of either type B or C.

DISCUSSION

The results of our ultrastructural studies support previous immunological and biochemical investigations which indicated a certain similarity of the two new virus isolates, M432 and M832, to known retroviruses of type B but, at the same time, provided sufficient data to justify their classification into a new class of retroviridae (Callahan et al. 1976).

In this report we have shown that cells infected with the two new isolates release virions by budding of intracytoplasmic, doughnut-shaped virus precursors resembling type A particles at their cell membranes. Virus release of such a kind is characteristic for retroviruses of type B as exemplified by the mammary tumour and guinea pig agents (Bernhard, 1973; Dahlberg et al. 1974; Michalides et al. 1975). The resemblance to type B viruses is further characterized by a certain likeness of the mature virions of one isolate, M432, to
already known type B virions since both exhibit characteristic spikes on their surface, a finding that was overlooked in a previous publication (Callahan et al. 1976).

On the other hand, certain morphological criteria of the newly isolated virions as well as their interactions with the cells indicate differences to known viruses of type B: (1) in the mature virions of the isolates studied here, the nucleoids are positioned centrally whereas in other type B virions the nucleoids are located excentrically, a criterion used for many years to distinguish type B virions from other retroviruses. (2) The number of intracytoplasmic type A particles is usually much higher in cells of mouse mammary tumours known to be infected with virus of type B than in cells infected with our isolates, since, in those cases, large accumulations of particles can be seen in different areas throughout the cytoplasm. This phenomenon was never observed in our material. (3) A comparison between virus precursors, the type A particles, of the new isolates with those of known type B viruses revealed distinct differences in regard to their staining behaviour in ultrathin sections. In previously described immature viruses of type B, two layers comprising the 'doughnut' can be resolved clearly due to differences in their staining affinity for heavy metals. In type A particles of the new isolates, recognition of these two layers is difficult since they exhibit rather similar affinities to heavy metal stains. (4) Unlike virions of type B and M432, virions of isolate M832 do not exhibit surface spikes of highly ordered arrangement. This property permits a morphological differentiation between isolate M432 and M832.

It is well known, that type A particles of the mammary tumour agent and their precursors, crescent-shaped forms, appear first adjacent to the Golgi areas within a granular matrix (Moore, 1962). Recent investigations support the assumption that type A particles found in cells infected with the mammary tumour agent represent pre-formed mammary tumour capsid structures (Tanaka, 1977; G. Smith, personal communication). In our material, the obvious similarity of the intracytoplasmic type A particles with the cores of budding and extracellular virions, both in regard to size and appearance, suggests that both represent a step in the development of the mature virion: crescent forms being precursors of type A particles and these, in turn, precursors of the mature virions. The maturation of the virions described here resembles therefore, in some aspects, the development of the mammary tumour virus and the guinea pig agent, both classified as retroviruses of type B (Dalton et al. 1974).

Little is known about the intracellular transport of virus precursors to the cell surface where they are enveloped by plasma membranes. The investigations described here give, for the first time, electron microscopic evidence of the close apposition of microtubules with virus precursors of type A in interphase cells. Microtubules are known to represent an elaborate tubular network (cytoskeleton) in the cytoplasm which is thought to be a major intracellular transport mechanism of secretory granules and other cellular components (Ehrlich & Bornstein, 1973; Lacy et al. 1968; Lemarchand et al. 1973; Porter, 1973; Sasaki & Tashiro, 1976). The close association of microtubules with virus precursors as seen in interphase cells of our preparations suggests that microtubules may play a role in transportation of the virus precursors to the cell membrane. Supporting this concept are our findings that virus precursors accumulate in cells arrested in metaphase at the initiation sites of microtubules. In this context it is noteworthy that movement of the virus precursors from the kinetochore region was observed again concomitantly with the reassembly of microtubules in the cytoplasm in late stages of the mitotic cycle.

Close association between centrioles and virus-like particles of type A has been observed previously by Wheatley (1974) and Gould & Borisy (1976, 1977) in interphase cells of the chinese hamster ovary (CHO) cell line grown in vitro.
Brinkley (personal communication) has confirmed this observation and, in addition, found such particles close to centrioles in cells of the PTK I line. The data presented here, for the first time, give evidence that lirosions belonging to the family of Retroviridae can be related to centriole-associated virus precursors. Since centrioles are known to be self-replicating organelles, being equally divided among daughter cells, it is intriguing to speculate that this association could then represent a possible mechanism for the vertical transmission of these viruses. This possibility was previously discussed by Wheatley (1974) with respect to his observations on CHO cells.

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REFERENCES


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