Intracellular Type A Retrovirus Movement Associated with an Intact Microtubule System

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SUMMARY

Intracytoplasmic type A particles known to be precursors to type B retroviruses in murine, hamster and marsupial cells are closely associated with microtubules and microtubule organizing centres. In this publication, the active participation of microtubules in the intracellular transport of the particles to the cell surface has been examined in NIH 3T3 cells infected with M432 virus using vincristine sulphate (VCR) as inhibitor of microtubule polymerization. The release of virus at different times after exposure to VCR was quantified by reverse transcriptase determinations of cell supernatants and by electron microscopic quantification of the number of virions at the cell surface using freeze-dried whole cell replicas. These studies indicate that VCR inhibits both microtubule polymerization and virus release, and thus suggest that intact cytoplasmic microtubules are necessary for intracellular transport and release of virus.

INTRODUCTION

Intracytoplasmic type A particles known to be precursors to certain type B viruses (Table 1), are endogenous to murine, hamster and marsupial cells. A number of these particles are characterized by their close association with the cytoskeleton, especially the microtubular system, insofar as they and their crescent-shaped precursors accumulate preferentially at microtubule organizing centres (MTOC) (Wheatley, 1974; Heine & Todaro, 1978; Calafat & Hilkens, 1978). In interphase cells, most particles are clustered at MTOC around centrioles and a few have been seen in close proximity to microtubules (Snyder & McIntosh, 1976). In cells arrested in metaphase by low concentrations of the microtubule inhibitor Colcemid, type A particles accumulate at MTOC of individual chromosomes, i.e. centromeric regions expressing kinetochores (Gould & Borisy, 1976; Heine et al., 1979a). A quantitative study of chromosomes isolated from Colcemid-treated CHO cells carrying such endogenous particles showed that type A particles migrated from cytoplasm to kinetochore regions. In fact, type A particles were preferentially located on kinetochores that retained microtubules in the presence of Colcemid (Heine et al., 1979b). Additional support for the affinity of these type A particles to the microtubular system is provided by experiments in which the microtubule-depolymerizing agent vincristine sulphate (VCR) was used (Heine et al., 1979b). Under these conditions, type A particles are found to associate preferentially with para-crystals of tubulin which form in the cytoplasm under the influence of the drug.

Intracytoplasmic type A particles must move from the cytocentre to the cell surface where they acquire an enveloping membrane and are released by a budding process (Heine et al., 1979a). At the present time, little is known about the forces responsible for the intracellular transport of virions and their precursor particles. The observations discussed in the preceding paragraph imply an active participation of the microtubular system in such movements.
Table 1. Occurrence of MTOC-associated intracytoplasmic type A particles

<table>
<thead>
<tr>
<th>Family</th>
<th>Cell line</th>
<th>Type A particle origin</th>
<th>Recovery of extracellular virus</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cricetidae</td>
<td>CHO</td>
<td>Endogenous</td>
<td>-</td>
<td>Wheatley (1974)</td>
</tr>
<tr>
<td></td>
<td>CHO</td>
<td>Endogenous</td>
<td>+</td>
<td>Heine et al. (1979a)</td>
</tr>
<tr>
<td></td>
<td>CHL</td>
<td>Endogenous</td>
<td>+</td>
<td>Calafat &amp; Hilkens (1978)</td>
</tr>
<tr>
<td>Muridae</td>
<td>NIH 3T3</td>
<td>Mus cervicolor</td>
<td>+</td>
<td>Callahan et al. (1977)</td>
</tr>
<tr>
<td></td>
<td>SC-1</td>
<td>M. caroli</td>
<td>+</td>
<td>Callahan et al. (1977)</td>
</tr>
<tr>
<td></td>
<td>NIH 3T3</td>
<td>M. caroli</td>
<td>+</td>
<td>Heine &amp; Todaro (1978)</td>
</tr>
<tr>
<td></td>
<td>Swiss 3T3</td>
<td>Endogenous</td>
<td>-</td>
<td>Albrecht-Buehler &amp; Bushnell (1980)</td>
</tr>
<tr>
<td>Heteromyidae</td>
<td>SCL 235</td>
<td>Endogenous</td>
<td>+</td>
<td>Hamilton et al. (1979)</td>
</tr>
<tr>
<td></td>
<td>CSL 227</td>
<td>Endogenous</td>
<td>+</td>
<td>Hamilton et al. (1979)</td>
</tr>
<tr>
<td></td>
<td>PtK-1</td>
<td>Endogenous</td>
<td>-</td>
<td>Pepper &amp; Brinkley (1977)</td>
</tr>
</tbody>
</table>

Tubules have long been regarded as a major factor in the intracellular transport of a variety of cell inclusions, such as vacuoles, granules, ribosomes and mitochondria (Freed & Liebowitz, 1970; Travis & Allen, 1981; MacGregor & Stebbings, 1970; Porter, 1973), but their exact role in this process is as yet not well understood. As indicated by the studies cited above, the type A particle-cell system may represent an excellent model to investigate the role of microtubules in intracellular virus particle movement.

In the present report, we have used the microtubule inhibitor VCR (Bensch & Malawista, 1969) to study the role of microtubules in virus release in control and microtubule inhibitor-treated NIH 3T3 cells infected with the murine M432 virus. As previously shown, type A viral precursor particles are abundant at MTOC in this model system (Heine & Todaro, 1978) and virions are released in large numbers at the cell surface under standard cell culture conditions (Callahan et al., 1976, 1977). Virus release at the cell surface was used as an indicator of undisturbed intracellular particle movement and was measured by direct particle counts at the cell surface and by reverse transcriptase activity in tissue culture supernatants. We also attempted to characterize the biochemical basis for the association between type A particles and microtubules by examining the distribution of tubulin and actin in these cells by immunoelectron microscopic methods. Previous reports have, in fact, indicated the presence of tubulin in the matrix of such type A particles (Pepper & Brinkley, 1977).

In this communication, we give the first evidence that an intact microtubular system is a prerequisite for the intracellular movement of these intracytoplasmic type A particles and their eventual release at the cell surface. At the same time, neither tubulin nor actin per se appears to be associated with the particles.

METHODS

**Virus, cell and culture conditions.** The M432 virus (obtained from G. J. Todaro) was isolated originally from spleen cells of *Mus cervicolor*, but subsequently grown in heterologously infected *M. musculus* cells, strain NIH 3T3 (Callahan et al., 1976). Morphological, biochemical and immunological characteristics of the infected cells have been described previously (Callahan et al., 1977). The infected, virus-releasing cells were grown in Falcon plastic dishes and maintained in Dulbecco’s MEM supplemented with 10% heat-inactivated foetal bovine serum, glutamine and antibiotics (maintenance medium).

**Treatment with vincristine sulphate.** Cells in subconfluent cultures were exposed for 1 h to various (10^{-5} to 10^{-7} M) concentrations of VCR (Oncovin; E. Lilly, Indianapolis, Ind., U.S.A.). In recovery experiments, cells were washed twice with Dulbecco’s phosphate-buffered saline (PBS) after exposure to the drug and refed with maintenance medium for various periods of time (1 to 24 h). At appropriate times, cell samples were selected for immunofluorescence studies, electron microscopic examination and for quantification of cell surface-bound virus particles; tissue culture supernatants were examined for reverse transcriptase activity.

**Reverse transcriptase determination.** The supernatant tissue culture fluids were harvested concomitantly and assayed for sedimentable RNA-dependent DNA polymerase (reverse transcriptase) activity using magnesium and manganese as the divalent cations, a polyriboadenylate template, an oligodeoxythymidylate as primer and [3H]TTP as substrate.

**Electron microscopy.** Fixation, embedding and staining procedures for routine transmission electron microscopic studies (Heine, 1969), as well as the preparation of cell surface replicas for the visualization of cell
surface-bound virus particles, have been reported (Demsey et al., 1977). For immunoelectron microscopic studies the EGS fixation and ferritin-bridge labelling methods developed by Willingham & Yamada (1979) were employed. Briefly, the primary fixative consisted of 0-2% glutaraldehyde and 1% ethyldimethylaminopropylcarbodiimide in phosphate buffer pH 7-0. Permeabilization of the cells was achieved by incubation in 0-05% saponin, 1 mM-EGTA and 4 mg/ml normal rabbit γ-globulin in Dulbecco's PBS. The same permeabilization buffer was used in all incubation steps which included: (i) affinity column-purified rabbit anti-tubulin antibody or normal rabbit globulin; (ii) goat anti-rabbit γ-globulin; (iii) affinity column-purified rabbit anti-horse spleen ferritin; (iv) horse spleen ferritin. Incubations were carried out at 23 °C, 1 h for each step. Unless stated otherwise, the antibodies used were obtained from Cappel Laboratories, Cochranville, Pa., U.S.A. Incubations were terminated by post-fixation for 15 min using 3% glutaraldehyde, followed by 1-5% OsO₄, both in PBS, dehydration in alcohol and embedding in Epon-Araldite. Ultrathin sections were stained with lead citrate (Reynolds, 1963) and bismuth subnitrate (Ainsworth & Karnovsky, 1972).

Indirect immunofluorescence. Cells on coverslips were fixed for 30 min in a 1:10 dilution of formaldehyde (J. T. Baker, Phillipsburg, N.J., U.S.A.) in PBS at room temperature. The fixed cells were then permeabilized with cold acetone for 7 min, air-dried and incubated with rabbit anti-tubulin serum (1:60 dilution in PBS) for 30 min at 37 °C. After a 10 min wash in PBS, cells were stained with a 1:60 dilution of rhodamine-conjugated goat anti-rabbit globulin (Cappel Laboratories). Cells were mounted in 5% glycerol and viewed with epifluorescence, ×63 objective NA 1-4, in a Leitz Ortholux II microscope. Fluorescent images were photographed with 35 mm Tri-X film (Kodak).

The percentage of cells with a decreased number of cytoplasmic microtubules was determined for 100 cells examined consecutively on each coverslip (see Table 3).

RESULTS

Vincristine inhibited budding of virus from the cell surface

As demonstrated previously (Demsey et al., 1977) and as shown in Fig. 1, budding virions can readily be visualized at the cell surface using a method for freeze-drying intact cells. Budding virions were easily recognized as structures studded with knob-like projections of approximately 10 nm in diameter. Virus particles were released in large numbers from control cells examined 1, 2, 3 and 24 h after medium change and were distributed regularly on the cell surface either as single particles or in small clumps (Fig. 1). After treatment with 10⁻⁵ M-VCR for 1 h cells had more villi and blebs at their surfaces. In spite of these cell surface changes, budding virions could be recognized easily, but occurred to a lesser degree in treated cells (Fig. 2). The decrease of virus budding was even more pronounced after a 2 h treatment using the same drug concentration (data not shown). Likewise, if cells were allowed to recover for 3 h following a 1 h treatment period, budding virus was greatly decreased at the cell surface (Fig. 2 and Table 2).

Vincristine inhibited extracellular reverse transcriptase activity

Reverse transcriptase determinations reflecting the number of virus particles present in the culture supernatant are illustrated in Fig. 3. Samples taken from cells during recovery after a 1 h exposure to high concentrations of VCR (10⁻⁵ M, 10⁻⁶ M) showed a marked reduction in enzyme production, resulting in the final activity of 15 to 30% of the control values. The lowest concentration of VCR (10⁻⁷ M) decreased extracellular reverse transcriptase initially, followed by a partial recovery to about 60% of the controls during a 24 h recovery period. These data support our morphological findings indicating a pronounced reduction in virus release immediately following the VCR treatment. The data are also in agreement with our immuno-histochemical findings that indicate a reversal of the drug's effect on microtubular arrangement only after exposure to the low dose of 10⁻⁷ M-VCR.

Table 2. Estimation of the effect of VCR on virus release at the cell surface by direct particle counting

<table>
<thead>
<tr>
<th></th>
<th>24 h MM</th>
<th>1 h 10⁻⁵ M-VCR</th>
<th>1 h 10⁻⁷ M-VCR</th>
<th>3 h MM</th>
<th>3 h MM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus particles/unit area</td>
<td>132</td>
<td>117</td>
<td>20</td>
<td></td>
<td>153</td>
</tr>
<tr>
<td>Standard error</td>
<td>63:7</td>
<td>67:2</td>
<td>23</td>
<td></td>
<td>86:1</td>
</tr>
</tbody>
</table>

* MM, Maintenance medium.
Fig. 1. Carbon/platinum replica of freeze-dried NIH 3T3 cell surface infected with M432 virus (control). Budding viruses are numerous (arrow). Bar marker represents 1 μm. Inset: virus bud with knob-like surface. Bar marker represents 100 nm.

Fig. 2. Cell preparation as in Fig. 1 following 10⁻⁵ M-vincristine sulphate (VCR) treatment for 1 h and recovery in maintenance medium for 3 h. Virus buds are infrequent (arrow). Bar marker represents 1 μm.

![Graph showing sedimentable reverse transcriptase activity](image)

**Fig. 3.** Sedimentable reverse transcriptase activity (c.p.m.) after treatment with VCR for 1 h and subsequent recovery in maintenance medium. Assay reflects incorporation of [³H]TTP employing poly(rA)-oligo(dT) as template and primer. VCR concentrations (M) were: 10⁻⁵ (△), 10⁻⁶ (▲) or 10⁻⁷ (○). ●, Control.

**Vincristine caused the disappearance of cytoplasmic microtubules**

Using indirect immunofluorescence with anti-tubulin antibody to detect changes in cytoplasmic microtubules, we found that a 1 h exposure to high concentrations of VCR (10⁻⁵ to 10⁻⁶ M) caused the complete disappearance of microtubules without recovery, while a 1 h treatment with a lower concentration (10⁻⁷ M-VCR) allowed recovery of microtubules by 6 h (Fig. 4). The percentages of cells with intact microtubules are tabulated versus time after VCR treatment in Table 3.
Retroviruses and the microtubule system

Fig. 4. Repolymerization of microtubules in cells treated with $10^{-7}$ M-VCR, but not in cells treated with $10^{-5}$ M-VCR for 1 h. (a) Control cells, untreated. (b) Cells treated with $10^{-5}$ M-VCR for 1 h followed by a 3 h recovery in maintenance medium: no microtubule recovery. (c) Cells treated with $10^{-7}$ M-VCR: after 3 h recovery, some cells show microtubules partially repolymerized. Small arrows delineate cell membrane; arrowhead shows periphery of microtubules. (d) Cells treated with $10^{-7}$ M-VCR for 1 h: after 5-5 h recovery, some cells show complete recovery of cytoplasmic microtubules. Bar marker represents 10 μm.
Table 3. *Time course of microtubule recovery from 1 h treatment of VCR*

<table>
<thead>
<tr>
<th>Time after VCR treatment (h)</th>
<th>A† VCR concentration (M)</th>
<th>B‡ VCR concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>10⁻⁶</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5.5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* *Hundred cells were examined for each time point.
† 'Intact microtubules': include such cells whose cytoplasmic microtubules extend to the cell periphery.
‡ 'Intact microtubules': include cells whose cytoplasmic microtubules are present at least halfway to the cell periphery.

Indirect immunoelectron microscopy demonstrated no tubulin associated with intracytoplasmic type A particles

Using a fixation technique controlled for intracytoplasmic penetration of IgG (Willingham & Yamada, 1979) in NIH 3T3 cells, the localization of tubulin at microtubules and MTOC could
be demonstrated by electron microscopy. As illustrated in Fig. 5 and 6, microtubules were specifically bound by antibody to tubulin, as visualized by the ferritin-bridge technique. However, significant labelling of associated type A particles was not observed. Precursors to type A particles and extracellular, mature virions not associated with the microtubular system were also not labelled (Fig. 7, 8). Likewise, labelling experiments using an antibody to actin gave negative results with respect to the localization of actin at viral particles (results not shown).

DISCUSSION

Microtubules are ubiquitous components of the cell localized primarily in the cytoplasm and are implicated in a wide variety of cellular functions. They constitute part of the cytoskeleton, contribute to external cell movements and the intracellular transport of various cytoplasmic inclusions, and are necessary to maintain integrity and localization of cellular organelles.

Microtubules are also important for the replication of different viruses. During early stages of adenovirus infection, microtubules may facilitate directional movement of the infecting particles by guiding them through the cytoplasm towards the nucleus (Dales & Chardonnet, 1973). During their replicative phase, reoviruses are associated indirectly with microtubules as both are connected to a network of vimentin intermediate filaments. In this model system, disruption of microtubules also alters the intracellular distribution of the cytoplasmic virus factories; however, the viral yield is not affected, thus suggesting a less direct role of microtubules in viral replication (Sharpe et al., 1982). The number and distribution of microtubules are also profoundly changed in cells infected with frog virus 3. It is postulated that in this case microtubules are used in conjunction with other cytoskeletal elements to maintain virus assembly sites at their proper location inside the cell (Murти & Goorha, 1983).

The role of microtubules in the replicative cycle of the enveloped retroviruses is not yet clear and may vary from one virus system to the other. In vitro, Moloney murine leukaemia virus production is severely inhibited when microtubule-depolymerizing drugs are present in the culture medium. As production of viral proteins is not reduced under these experimental conditions, it may be concluded that an unperturbed microtubule system is necessary for the movement of viral proteins to the cell surface where virus particle assembly occurs (Satake & Luftig, 1982). Likewise, association of the p27 Rous sarcoma virus internal protein with microtubules has been suggested (Stanislawski, 1983).

The present investigation shows that microtubules are an absolute prerequisite for the intracytoplasmic movement of M432 type A particles to the cell surface where virus particles are released by budding from the plasma membrane. These type A particles are known to be precursors to a retrovirus and have been classified as belonging to a new class of murine type B viruses (Callahan et al., 1976). However, by morphological characterization, the particles are also members of a unique group of enveloped viruses (see Table 1). All members of this group are distinguished by the close association of their intracellular precursor, the type A particle, with microtubules and/or MTOC. This holds true for MTOC at centrioles in the cytoplasm of interphase cells and, as far as studied, for MTOC at kinetochores of chromosomes during mitosis (Heine et al., 1980). In addition, these type A particles and their mature, extracellular forms differ in size and fine structure from the classical retroviruses of the genus B, of which the murine mammary tumour virus is the prototype (Bernhard, 1958).

Our observations are in agreement with recently published findings (see preceding paragraphs) that underscore the role of a functioning microtubule network in virus assembly and the directional movement of (virus precursor) particles in the cytoplasm. However, the nature of the linkage between microtubules and virus particles is still uncertain. Although the findings of Pepper & Brinkley (1977), showing the presence of tubulin on the surface of type A particles in CHO cells, indicate a direct linkage, neither tubulin nor actin could be identified on the surface of M432 type A particles in our immunoelectron microscopic studies. Further progress in the understanding of this linkage phenomenon awaits the characterization of the underlying biochemical and/or biophysical factors.

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REFERENCES


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