Identification of Type C Viruses by Electron Microscopy

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ABSTRACT

The cores of a small proportion of the particles in purified preparations of type C viruses are characterized by two concentric shells that have a "double-ring" appearance in specimens prepared for electron microscopy by negative staining or thin sectioning techniques. This distinctive feature allows such particles to be recognized as viral in impure preparations. Detectability of double-ring particles is considerably enhanced by treating the specimens with a non-ionic detergent before fixation and negative staining. The limit of detectability of type C virus (identified by the double-ring feature) in the presence of a large excess of nonviral particulates of similar size and density was assessed.

INTRODUCTION

Type C viruses in unpurified or semipurified preparations present problems in identification by electron microscopy that are not encountered with many other viruses. They do not show, by standard methods of contrast enhancement, specific viral characteristics that allow individual particles to be positively identified and clearly distinguished from nonviral particulates.

Preparations of type C virus particles invariably have associated with them a proportion of particles with a morphology different from that of the majority. They are referred to as "enveloped type A" (4) particles and are generally believed to represent particles that have not completed the extracellular maturation process (2, 3, 6, 7). As seen in fixed, negatively stained preparations, enveloped type A particles consist of a core in the form of a double ring (concentric spherical shells in 3 dimensions) surrounded by a membranous envelope. The inner shell probably consists of a viral nucleoprotein complex, and the outer represents the viral capsid. The double-ring core allows these particles to be clearly distinguished from nonviral particulates and appears to be a reliable diagnostic feature.

We describe here experiments in which the detectability of double-ring particles was enhanced by treating viral preparations with the non-ionic detergent NP40 before fixation and negative staining. As previously reported by Frank (6), we found that this treatment resulted in an apparent increase in the proportion of ring-like particles. The reason for this apparent increase after detergent treat-
The corresponding negatively stained preparation, consists shown in Fig. 1b, however, the proportion of naked cores is shown in Fig. 2b. The detergent-treated preparation, like treatment and processing the gel for thin sectioning, as described in "Materials and Methods." The results are some disorganized filamentous material. A small proportion particles, naked angular cores, and sheets of membranous essentially of 3 types of structure, enveloped double-ring acetate after fixation in 3.5% glutaraldehyde with and with increased. Double-ring particles constituted considerably increased. Double-ring particles constituted percentage of double-ring particles is observed after detergent treatment. The envelopes of the double-ring particles seem to be relatively unaffected by the detergent, in contrast to particles with angular cores, the envelopes of which are converted into membranous sheets with release of cores.

The selective preservation and easy visibility of double-ring particles after exposure to detergent suggested that this treatment might be of value in improving the detectability of virus in impure preparations. Simulated impure preparations of virus, made by mixing known numbers of virus particles with nonviral particulates of similar size and density obtained from FBS, were examined: (a) after trypsin treatment and (b) after detergent treatment, followed in each instance by fixation in glutaraldehyde and negative staining with uranyl acetate. Fig. 3a shows a typical field from a trypsin-treated preparation of FBS particles (without added virus). Two main types of particles are present: (a) round particles 60 to 80 nm in diameter with prominent surface projections (Fig. 3a, inset) and (b) membrane-bound particles with a diameter of about 150 nm; these are more pleomorphic than are the first type and have no distinct surface projections; some are penetrated by stain but show no distinct internal structure. No particles resembling the double-ring type of M4 virus particles were observed. Fig. 3b shows a typical field from a similarly treated specimen consisting of a mixture of equal volumes of purified M4 virus and a FBS particle suspension. Double-ring particles (broad arrow) and particles with angular cores (crossed arrow) are readily distinguishable from the first type of FBS particle (fine arrow), but there are other particles, including some not penetrated by the stain, that cannot be classified definitely as viral or the second type of FBS particle. A field representative of a similar mixture of M4 virus and FBS particles treated with NP40 before fixation and negative staining is shown in Fig. 3c. Double-ring particles (broad arrows) are easily identified, standing out clearly against a background consisting mainly of membranous sheets and fragments. In similarly treated preparations of FBS particles without added M4 virus, no particles of double-ring morphology were detected.

Using this method the limit of detectability of double-ring particles (serving as a marker for M4 virus) was assessed by examination of mixtures consisting of equal volumes of a FBS particle preparation and a purified M4 virus preparation at increasing dilutions of the latter. At lower dilutions double-ring particles were scored on plates taken at a magnification of ×15,000 of fields selected at random. At the highest dilution random sampling was not feasible and particles were scored by scanning a much larger area directly on the screen of the electron microscope. The results are shown in Table 1. In each instance the number of double-ring particles detected was in reasonable agreement with, although somewhat smaller than, the number expected. The latter figure was based on the number of double-ring particles observed in negatively

RESULTS

M4 virus particles were negatively stained with 1% uranyl acetate after fixation in 3.5% glutaraldehyde with and without prior treatment with 0.25% trypsin. The appearances were essentially the same except that after trypsin treatment the viral envelopes were more consistently penetrated by the stain. A typical field showing M4 virus negatively stained after trypsin treatment and fixation is illustrated in Fig. 1a. In nearly every instance the viral envelope, which appears as a white halo, has been penetrated by the stain, revealing the internal core. Most of the cores have a somewhat angular outline and show little fine structure except for some disorganized filamentous material. A small proportion of the cores (black arrows) have a more regular structure, which appears as 2 concentric rings enclosing a partially stain-filled central area. The appearance of M4 virus particles treated as described corresponds quite closely (but with contrast reversed) to that of particles seen in thin sections of virus pellets (Fig. 2a). The percentage of particles with concentric double-ring cores (about 15%) was the same by both methods. The appearance of these particles suggests strongly that they correspond to the "immature" particles that are observed in association with virus-producing cells (3, 4).

The morphology of the virus particles after treatment with the detergent NP40 (0.1% for 1 min) and subsequent fixation and negative staining was strikingly different. The majority of the particles were then of the double-ring type (Fig. 1b). Enveloped particles with angular cores, which constituted the majority of the trypsin-treated preparation, were no longer observed. Instead there were large sheets of membranous material and small numbers of scattered, naked angular cores (Fig. 1b).

The greatly increased proportion of double-ring particles in the negatively stained preparations after detergent treatment could be due to the specimen preparation procedure used, since this did not ensure that the specimens viewed in the electron microscope were representative of the virus preparation. Selective loss of material from the grid or differential adsorption of particles to the substrate might have occurred. This uncertainty was avoided and improved sampling of the virus preparation was obtained by immobilizing the virus material in agar gel immediately after detergent treatment and processing the gel for thin sectioning, as described in "Materials and Methods." The results are shown in Fig. 2b. The detergent-treated preparation, like the corresponding negatively stained preparation, consists essentially of 3 types of structure, enveloped double-ring particles, naked angular cores, and sheets of membranous material. Compared with the negatively stained preparation shown in Fig. 1b, however, the proportion of naked cores is considerably increased. Double-ring particles constituted 18% of the total particles (enveloped double rings plus angular cores), a value only slightly higher than the 15% double-ring particles observed in the untreated control preparation (Fig. 2a). Thus, it appears that, under conditions in which virus material is conserved and representative specimens are examined, no significant increase in the percentage of double-ring particles is observed after detergent treatment. The envelopes of the double-ring particles seem to be relatively unaffected by the detergent, in contrast to particles with angular cores, the envelopes of which are converted into membranous sheets with release of cores.

The selective preservation and easy visibility of double-ring particles after exposure to detergent suggested that this treatment might be of value in improving the detectability of virus in impure preparations. Simulated impure preparations of virus, made by mixing known numbers of virus particles with nonviral particulates of similar size and density obtained from FBS, were examined: (a) after trypsin treatment and (b) after detergent treatment, followed in each instance by fixation in glutaraldehyde and negative staining with uranyl acetate. Fig. 3a shows a typical field from a trypsin-treated preparation of FBS particles (without added virus). Two main types of particles are present: (a) round particles 60 to 80 nm in diameter with prominent surface projections (Fig. 3a, inset) and (b) membrane-bound particles with a diameter of about 150 nm; these are more pleomorphic than are the first type and have no distinct surface projections; some are penetrated by stain but show no distinct internal structure. No particles resembling the double-ring type of M4 virus particles were observed. Fig. 3b shows a typical field from a similarly treated specimen consisting of a mixture of equal volumes of purified M4 virus and a FBS particle suspension. Double-ring particles (broad arrow) and particles with angular cores (crossed arrow) are readily distinguishable from the first type of FBS particle (fine arrow), but there are other particles, including some not penetrated by the stain, that cannot be classified definitely as viral or the second type of FBS particle. A field representative of a similar mixture of M4 virus and FBS particles treated with NP40 before fixation and negative staining is shown in Fig. 3c. Double-ring particles (broad arrows) are easily identified, standing out clearly against a background consisting mainly of membranous sheets and fragments. In similarly treated preparations of FBS particles without added M4 virus, no particles of double-ring morphology were detected.

Using this method the limit of detectability of double-ring particles (serving as a marker for M4 virus) was assessed by examination of mixtures consisting of equal volumes of a FBS particle preparation and a purified M4 virus preparation at increasing dilutions of the latter. At lower dilutions double-ring particles were scored on plates taken at a magnification of ×15,000 of fields selected at random. At the highest dilution random sampling was not feasible and particles were scored by scanning a much larger area directly on the screen of the electron microscope. The results are shown in Table 1. In each instance the number of double-ring particles detected was in reasonable agreement with, although somewhat smaller than, the number expected. The latter figure was based on the number of double-ring particles observed in negatively
stained specimens of the original preparation of M4 virus. On the basis of these results, it was concluded that: (a) most double-ring particles are detectable after detergent treatment even in the presence of a great excess of nonviral particulates of similar dimensions and (b) the limit of detectability corresponds to a concentration of M4 particles of about $5 \times 10^8$/ml (Table 1).

### DISCUSSION

The particles here described as the double-ring type are morphologically similar to the budding and newly released virus particles observed at the surface of virus-producing cells and referred to as enveloped type A (4) or immature type C (3) particles. The biological activity of these particles and their precise relationship to "mature" type C particles, which constitute the majority of most infectious virus preparations, is still not clear (5). The morphological changes that occur after the newly formed particles are released from the cell are accompanied by changes in conformation of the viral protein and RNA (2, 7). In the avian myeloblastosis virus system, Korb et al. (7), using virus harvested at intervals ranging from 3.5 min to 17 hr, obtained good correlation between morphological change from ring-like to condensed core configuration and conversion of viral RNA from 30 to 40S subunits to 60 to 70S RNA. Virus harvested at the shortest time interval (3 to 5 min) had a high proportion (~60%) of ring-like forms. This proportion decreased steadily for virus harvested at longer time intervals and reached a plateau of about 15% at the longest time interval (17 hr). These morphological and conformational changes have generally been interpreted as an indication of an extracellular maturation process leading to the formation of mature biologically active virus. Smith (9), working with avian sarcoma virus, obtained evidence in favor of this concept by showing that the specific activity of the virus (measured by the ratio of particles to focus-forming units) increased as the harvest interval was increased from 30 sec to 20 min. At longer harvest intervals specific infectivity decreased, the decline being attributable to heat activation of the virus.

On the other hand, De Harven (4) has criticized the extracellular maturation concept and has suggested that enveloped type A particles may be biologically active particles and that so-called mature type C particles may be not only structurally degenerate but also functionally impaired. The maturation concept has also been brought into question more recently by Frank (6), who studied the effect of detergent on a murine oncovirus (Friend virus). He found that particles with round inner bodies (enveloped type A particles), after treatment with the detergent NP40 and negative staining with uranyl acetate, represented 50 to 80% of the total particles. This was greatly in excess of the 3 to 5% of such particles observed in preparations negatively stained without detergent treatment or fixed in glutaraldehyde before being treated with detergent and negatively stained. These results were interpreted as indicating a real increase in the number of particles with ring-like cores brought about by the action of the detergent. The detergent was thought either to have a direct preservative effect on the ring-like cores or to act indirectly by increasing the permeability of the envelope to the uranyl acetate stain, which had a stabilizing effect on the cores. This interpretation implies that most or all of the particles with angular cores (mature type C particles) observed by standard techniques are poorly preserved and structurally altered because of inadequate stabilization of the cores. Frank's explanation appears to be ruled out by the experiment illustrated in Fig. 2b in which no significant increase in the proportion of particles with ring-like cores was observed after detergent treatment. Angular-cored particles were still present in expected numbers, although they were stripped of envelope by the detergent treatment. Thus, there is no reason to believe that the condensed angular cores observed within particles in thin sections processed by the usual procedures or in standard negatively stained preparations are artifacts caused by inadequate preservation.

Whatever the roles and biological activities of the 2 classes of type C viruses, it remains true that the immature particles are more uniform and regular in structure than are the mature particles, which are pleomorphic and lacking in regularity. Thus, the former particles are more readily recognized as viral than are the latter, which may be confused with nonviral particulates such as those com-

### Table 1

<table>
<thead>
<tr>
<th>Preparation</th>
<th>No. of M4 particles per ml</th>
<th>Expected no. of double-ring particles per unit area</th>
<th>Observed no. of double-ring particles per unit area</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4</td>
<td>$2 \times 10^{11}$</td>
<td>20</td>
<td>30 (av. of 9 plates)</td>
</tr>
<tr>
<td>M4 + FBS</td>
<td>$10^{10}$</td>
<td>1</td>
<td>9 (av. of 20 plates)</td>
</tr>
<tr>
<td>M4 (1/20) + FBS</td>
<td>$5 \times 10^{10}$</td>
<td>0.75</td>
<td>0.4 (av. of 48 plates)</td>
</tr>
<tr>
<td>M4 (1/200) + FBS</td>
<td>$5 \times 10^8$</td>
<td>0.075</td>
<td>0.02 (av. of about 1250 plate equivalents)</td>
</tr>
</tbody>
</table>

*Based on plates taken of specimens prepared by negative staining after NP40 treatment, except for the purified M4 preparation, which was treated with trypsin and negatively stained.

*Estimate based on determination of protein concentration of purified M4 preparation.
monly present in sera and cell culture media. Treatment of impure virus preparations with non-ionic detergent has the further advantage that it disrupts nonviral membrane-bound particles and uncoats mature type C particles, leaving the immature particles apparently unaffected. The characteristic double-ring appearance of the latter renders them readily recognizable in negatively stained preparations, even in the presence of a great excess of nonviral material. The results of the mixing experiments shown in Table 1 indicate that the limit of detectability of type C particles, as determined by the presence of identifiable double-ring particles, is about $5 \times 10^8$ particles/ml. Since an estimated thousand-fold concentration was achieved during purification, this value corresponds to $5 \times 10^8$ particles/ml in the culture medium from which the virus was purified.

All preparations of type C viruses that we have examined, including commercially obtained, purified preparations of viruses of avian, murine, feline, and simian origin, contain a proportion of double-ring particles that are readily recognizable by negative staining, especially after detergent treatment. The percentage varies from 1 preparation to another but, when assessed by standard thin sectioning or negative staining techniques, is usually in the range of 5 to 15%. If human counterparts to known type C viruses exist, it is to be expected that they also contain a proportion of double-ring particles. From our experiments we conclude that the detection of such particles by negative staining after nonionic detergent treatment offers the most promising method for demonstrating unequivocally the presence of type C particles in human materials such as leukemic plasma or media in which leukemic cells have been cultured. Although using double-ring particles as a marker for type C viruses decreases the sensitivity of detection by a factor of about 10, this is more than offset by the enhanced ease and reliability with which these particles can be identified.

REFERENCES

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