An Electron Microscopic Study of Lactic Dehydrogenase Virus in Cultures of Mouse Peritoneal Macrophages

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SUMMARY

An electron microscopic study was made of the plasma lactic dehydrogenase elevating virus of Riley et al. (1960) in cultures of mouse peritoneal macrophages. Four morphologically different forms of particle were seen: (1) large round particles, 510 Å in diameter, consisting of a nucleoid enclosed by dense double membranes (310 Å) and a less dense halo, which was occasionally seen to be bounded by a thin external membrane; (2) large elongated particles of similar morphology to type 1; (3) small round particles, 330 Å in diameter, very similar in appearance to the nucleoid of the large particle; (4) small rod-shaped particles, also surrounded by double membranes and varying in length between 640 and 1240 Å and in width between 200 and 380 Å. Similar particles were observed in pellets prepared by ultracentrifugation from viraemic mouse plasma and the medium of infected cultures.

INTRODUCTION

The plasma lactic dehydrogenase elevating virus (LDH virus) of Riley et al. (1960) has been shown in electron micrographs of plasma pellets by several workers (Crippens & Burns, 1964; De Thé & Notkins, 1965; Du Buy & Johnson, 1966; de Harven & Friend, 1966) whose reports of size and shape vary. In view of the high virus titre attained in cultures of mouse peritoneal macrophages infected in vitro (Evans & Salaman, 1965) an attempt was made to demonstrate intracellular particles by electron microscopy. Forms of particles not previously described were observed.

METHODS

Plasma lactic dehydrogenase elevating virus was obtained from the plasma of Parkes mice, a randomly bred strain of albinos, infected 24 hr previously with stock virus, as described by Mahy et al. (1965). The preparation was titrated for infectivity as described by Rowson, Mahy & Salaman (1965), and was found to contain $10^7$ ID$_{50}$ LDH virus/ml. Before inoculation into cultures, the virus was diluted 1/9 with tissue culture maintenance medium (MM), which consisted of Eagle's basal medium plus 1% (v/v) calf serum. Stock normal plasma was obtained in a similar manner to

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the infected plasma. At the beginning of the experiment all mice were found to have normal levels of plasma lactic dehydrogenase activity, indicating that they were not carrying LDH virus.

The preparation of peritoneal macrophage cultures was described by Evans & Salaman (1965). Twenty-four-hour cultures were inoculated with 5 ml of MM containing 10⁶ ID50 LDH virus/ml. The inoculum was not washed off. Control cultures were inoculated with 5 ml MM containing a 1/9 dilution of stock control plasma. Three days later, the culture medium in each group was decanted, pooled and centrifuged at 10,000 rev./min. for 30 min. to deposit debris. The supernatant fluids were titrated for viral infectivity. All cultures were washed ten times with cold phosphate buffered saline, after which the cells were detached from the glass by means of a rubber policeman, since trypsinization was ineffective. The cells from each group were pooled, sedimented at 2000 rev./min. for 20 min. and the supernatant fluid removed. The cell pellets were fixed either in 1% (w/v) OsO₄ buffered at pH 7.4 according to Millonig (1961) or in 6.5% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer followed by Millonig's 1% (w/v) OsO₄. The cells were then dehydrated in graded concentrations of ethanol, stained with 0.5% (w/v) uranyl nitrate in methacrylate and embedded in methacrylate. Pellets of partially purified virus from plasma of mice infected with LDH virus 24 hr previously, and from uninfected mice, together with pellets from infected and control tissue culture medium, were prepared for electron microscopy in the same way. The procedure followed for the purification was described by De Thé & Notkins (1965) and Du Buy & Johnson (1965). Thin sections were cut on a Porter-Blum MT I microtome and examined in a Siemens Elmiskop I at 80 kV using an anticontamination trap. The cells were examined for virus at × 20000 magnification. Most measurements were made from prints at × 70000 magnification.

RESULTS

Four different types of particle, of which two predominated, were seen in the infected peritoneal macrophages: large round particles and small round or oval particles. Large elongated and small rod-shaped forms were seen less frequently.

The large, round particles (type 1) had an average external diameter of 510 Å (mean of 100 measurements ranging from 410 to 710 Å) of which 30% had a diameter of 500 Å. They had an electron-transparent core about 180 Å in diameter, surrounded by dense double membranes. This structure, referred to as the nucleoid, had an average diameter of 310 Å (range 200 to 480 Å). The average total thickness of the membranes was 58 Å (range 42 to 71 Å). The nucleoid was encircled by an electron-transparent halo 70 to 120 Å wide which appeared to be bounded by a thin membrane, though this was not always clearly visible (Pl. 1, fig. 1, 2).

Large elongated, almost rod-shaped particles (type 2) of similar morphology to type 1 were occasionally seen amongst the latter (Pl. 2, fig. 4).

The small round or oval particles (type 3) were very similar in appearance and dimensions to the nucleoid of the large particle. Their average external diameter was 330 Å (mean of 100 measurements ranging between 240 and 460 Å). These particles also had an electron-transparent core, approximately 200 Å in diameter, surrounded by double membranes of average total thickness 58 Å (range 42 to 71 Å) but these were less dense in appearance than those of the large, type 1, particle (Pl. 1, fig. 1, 2).
Electron microscopy of LDH virus

The fourth particle (type 4) was rod-shaped and was often seen in association with the small particles. The rods varied in length between 640 and 1240 Å and in width between 200 and 380 Å. They also showed an electron-transparent core enclosed by double membranes, averaging 58 Å in thickness (Pl. 1, fig. 3).

Particles were usually enclosed together, often in neat bundles, by a single membrane which was sometimes continuous with the endoplasmic reticulum. The bundles usually contained either groups of large (type 1) with occasionally large elongated (type 2) particles, or groups of small (type 3) and rod-shaped particles (type 4) (Pl. 1, fig. 1–3). However, all four types of particles have been seen in the same cell. Sometimes distinct groups of particles were enclosed by endoplasmic reticulum which formed the outer layer of the nuclear membrane. Occasionally groups of particles were also embedded in a dense amorphous substance, which made the correct identification of their type difficult (Pl. 1, fig. 1). Small and rod-shaped particles could occasionally be seen lying free in the cytoplasm (Pl. 2, fig. 6) and also surrounding vesicles. Groups of both small and rod-shaped particles were frequently seen to be tightly wrapped in another single membrane, rather like sausages in skins (Pl. 1, fig. 3). Virus particles were not evenly distributed in a large number of cells studied; in fact, few, if any, cells in each section examined contained particles. The cells in which virus was seen usually contained large numbers of particles and often showed changes consistent with cell death. As the LDH virus is so small, it is possible that cells containing only a few particles would be missed in an electron microscopic examination. Particles of similar morphology and dimensions to the four types occurring in the cell cultures, were observed in the partially purified pellets prepared from viraemic plasma. Most particles were of the large round type, having an average diameter of 440 Å. Elliptical forms varying from 450 to 750 Å in length and 180 to 370 Å in width occurred fairly frequently. These particles were not very distinct, and the outer halo was not always clearly visible so it was not possible to classify them. It may be that they represent particles which had become somewhat damaged by the centrifugation procedures. Some small (type 3) particles were also present. Rod-shaped (type 4) particles (Pl. 2, fig. 5) and elongated (type 2) particles were occasionally observed. Although many attempts were made to find particles in pellets prepared from the medium of infected cultures, in only a few pellets were particles discovered. This could be the result of a much lower concentration of LDH virus (10⁸ to 10⁹ ID₅₀/ml.). The particles in these pellets were of the same type as those occurring in the plasma pellets. None of the particle types described above was seen in the control cultures, medium or plasma.

DISCUSSION

Previous experiments (Evans, unpublished observations) showed the total intracellular virus concentration, after correction for dilution, to be approximately equal to the concentration of virus in the medium, i.e. between 10⁸ and 10⁹ ID₅₀/ml. Thus viral replication undoubtedly occurred. It seems unlikely that all the particles observed represent phagocytosed virus.

Our description of the structure and size of the particles occurring in the cultured cells and plasma pellets corresponds fairly well with those of others. Du Buy & Johnson (1966) described similar particles occurring in certain cells in the lymph nodes of mice infected with LDH virus. They reported a small particle consisting of a densely
staining annulus, about 250 Å in diameter, and a large particle, about 500 Å in diameter, consisting of a similar dense annulus with a poorly defined less dense halo.

De Thé & Notkins (1965) observed virus particles in thin sections of peritoneal macrophages taken from mice infected with LDH virus. The nucleoid was reported to be 260 to 290 Å in diameter with a dense shell, 70 to 90 Å wide, surrounded by an outer layer of low density, 50 to 70 Å wide, limited by what appeared to be a thin outer membrane. They also showed elongated forms and particles giving the appearance of maturation at the cell membrane of peritoneal macrophages. We did not observe budding from the cell surface in our infected macrophage cultures.

The dimensions reported for the LDH virus measured from electron micrographs of plasma pellets or ascitic fluid are shown in Table 1.

<table>
<thead>
<tr>
<th>Round particle (Å)</th>
<th>Elliptical particle (Å)</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 × 450</td>
<td>360 to 420 × 450 to 750</td>
<td>Crispens &amp; Burns (1964)</td>
</tr>
<tr>
<td>400</td>
<td>De Thé &amp; Notkins (1965)</td>
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</tr>
<tr>
<td>500</td>
<td>Du Buy &amp; Johnson (1965)</td>
<td></td>
</tr>
<tr>
<td>570</td>
<td>De Harven &amp; Friend (1966)</td>
<td></td>
</tr>
<tr>
<td>440</td>
<td>Prosser &amp; Evans (1967)</td>
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Our electron micrographs showing large numbers of particles in degenerating cells suggested the release of particles from disintegrated cells. The proportion of degenerating cells containing particles in the infected cell cultures was not significantly greater than the proportion of degenerating cells in the control cultures, so that it was difficult to determine whether a true cytopathic effect took place. Cytopathic effects were not observed by light microscopy (Evans & Salaman, 1965). Since all four types of particle have been identified in the plasma from which stock virus solutions were prepared, and in the culture cells and medium, at least one particle type must represent the mature, infective LDH virus. Notkins & Shocat (1963) and Rowson, Mahy & Salaman (1963) reported filtration experiments in which the LDH virus was retained by filter membranes of 740 and 670 Å respectively, but passed through those of 1100 Å. A particle size between 550 Å and 450 Å was calculated which favours the large particle as being infective. For, provided enough of the small 250 to 330 Å particles were present in the plasma, some infectivity would have been expected to pass through the smaller sized filter; that is, assuming the small particles seen in sections are not cross-sections of rod-shaped particles.

The possibility must be considered of the presence of two viruses, always associated together, only one of which is detected by the diagnostic tests used for the LDH virus. Experimental evidence for the existence of more than one type of virus particle in the plasma of mice infected with LDH virus has been presented by Adams & Bowman (1964), Crispens (1964) and Stark & Crispens (1965); though Du Buy & Johnson (1965) were unable to demonstrate two types of particles in their preparations. Recent immunological findings by Bailey et al. (1965), Rowson, Mahy & Bendinelli (1966) and Notkins et al. (1966), who showed that a fraction of the plasma virus is not neutralized by anti-LDH virus serum, also suggest the presence of particles with at least different antigenic properties.
We wish to thank Dr R. R. Dourmashkin, Dr R. J. C. Harris and Dr M. H. Salaman for help and advice during the course of this study, and Mrs Maureen Bedford and Miss Marie-Martine Bertholet for technical assistance.

REFERENCES


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EXPLANATION OF PLATES

Pls. 1 and 2 are electron micrographs of thin sections of mouse peritoneal macrophages infected with LDH virus *in vitro*. The bars represent 1000 Å.

**PLATE 1**

Fig. 1. A bundle of large round particles (type 1) (*LP*). Rod-shaped (type 4) and small particles (type 3) (*SP*) enveloped closely by a membrane. A group of particles (indicated by arrow) is embedded in a dense amorphous substance.

Fig. 2. Photographic enlargement of large particles (type 1).

Fig. 3. Photographic enlargement of small and rod-shaped particles closely enveloped by a membrane.

**PLATE 2**

Fig. 4. A group of large round particles (type 1) and large elongated particles (type 2).

Fig. 5. An electron micrograph showing small rod-shaped particles (type 4) in thin section of a pellet of partially purified virus prepared from plasma of LDH-virus-infected mice.

Fig. 6. Small round or oval particles (type 3) free in the cytoplasm of a cell.