Role of Lysosomes during Infection with Shope Fibroma Virus of Primary Rabbit Kidney Tissue Culture Cells

(Accepted 20 September 1973)

SUMMARY

At 30 to 60 min after infection of primary rabbit kidney cells with Shope fibroma virus, the majority of virus particles were found within dense granules identified as lysosomes, whereas by 24 h no particles could be observed. The lysosomes appeared to become more fragile as infection proceeded, cellular proteins being progressively released from 7 to 24 h. Extracted lysosomal enzymes had no effect on purified Shope fibroma virus.

It has been suggested (Allison, 1967) that lysosomes may play a role at several steps in virus replication in tissue culture cells. This role varies both with the virus and the cell line used. The penetration and replication of vaccinia virus were studied using the electron microscope (Dales, 1963, 1965; Dales & Kajioka, 1964), but the involvement of lysosomes was not described. In the present investigation, biochemical and electron microscopic techniques were used to study the possible involvement of lysosomes in the infection of susceptible cells by Shope fibroma virus (SFV), an oncogenic poxvirus.

Primary rabbit kidney cells in tissue culture were infected with SFV, OA strain (Shope, 1932), which had been purified from lysates of infected RK 13 cells (3 to 5 x 10^8 cells) by the technique described by Jacquemont, Precosta & Gautheron (1969). Radioactively labelled virus was obtained from infected cell cultures grown in medium containing [3H]-thymidine (sp. act. 18 Ci/m-mol; 0.2 μCi/ml; CEA France). Radioactivity was measured as previously described (Chardonnet & Dales, 1970) using a Packard ‘Tri-Carb’ model 3380 scintillation counter. Infectivity of virus was determined by c.p.e. on primary rabbit kidney cells (Jacquemont et al. 1972).

Adsorption of the SFV to cells, and its subsequent penetration was followed by electron microscopy. Synchronization of the process was achieved by allowing the SFV (10 ID/cell) to absorb to the cells at 4 °C for 2 h, then incubating the cells at 37 °C for the rest of the experimental period. Cell pellets were sectioned, and the cell-associated virus particles were counted as previously described (Chardonnet & Dales, 1970). Either a Siemens Elmiskop 101 or an AEI EM6B microscope was used at a tension of 80 kV.

At the end of the adsorption period at 4 °C virus particles were present only at the cell surface, sometimes in invaginations of the plasma membrane (Fig. 1). Some 60% of the inoculated virus could be shown to be associated with the cells either by particle counting, or by titration of the residual virus in the culture supernatant.

After 10 min incubation at 37 °C some particles remained at the cell surface (15 to 18%), a few were free in the cytoplasm (10 to 15%), most were in vacuoles close to the surface (60 to 65%), and some were in dense granules (10 to 14%) either alone or as small groups of two or three particles.

After longer periods of incubation (30 to 60 min) a few particles were still to be seen at the surface of the cell (5 to 10%), and some were free in the cytoplasm (10 to 20%); a number of these had lost their external envelope forming cores, particularly in the perinuclear region.
The majority of the particles (50 to 60%) were present, usually as groups of three or four, inside dense granules which were identified as lysosomes by staining for acid phosphatase using Gomori's technique (Chardonnet, Pruniéras & Sohier, 1962) (Figs. 2, 3).

Uninfected control cells and cells infected with SFV (1 ID/cell) for 1 h were fractionated as described previously (Chardonnet & Dales, 1970). The granular fraction consisting of mitochondria and lysosomes (ML) was isolated, and then separated by centrifuging through a sucrose density gradient. Fig. 4 (a, b, c) shows that the activity of three different acid hydrolases: acid phosphatase (EC. 3.1.3.2) assayed by the method of Chardonnet (1969), acid DNAse (EC. 3.1.4.6) assayed by the method of De Duve et al. (1955), and cathepsin D (EC. 3.4.4.23) assayed by the method of Gianetto & De Duve (1955), did not differ between the control and the infected cells. Enzyme activities are expressed in units, where 1 unit is the amount of enzyme required to degrade 1 μmol of substrate/min under the assay conditions (De Duve et al. 1955). In both cases there was a major peak of activity centred around a position corresponding to a density of 1.18 g/ml, and a second minor peak corresponding to a density of 1.10 g/ml. No release of lysosomal enzymes from the ML fraction into the supernatant fraction was observed. Total protein content as measured by the technique of Lowry et al. (1951) was also identical in the two cases (Fig. 4d). When labelled virus was used (Fig. 4e) two main peaks of radioactivity were observed: a peak corresponding to the position of lysosomes (57% of radioactivity) and a second at the bottom of the tube (including the pellet and the first two fractions), representing free virus. It was established that the
Fig. 4. Distribution on a sucrose density gradient (1.10 to 1.25 g/ml) of lysosomal enzymes, total proteins and SFV after centrifuging the ML fraction (mitochondria and lysosomes) derived from cells infected with 1 ID/cell of [3H]-thymidine labelled SFV for 1 h (△-△), or from control cells (○-○). Equilibrium sedimentation at 115 000g for 150 min. Units: acid phosphatase, n-mol/min/ml phosphate released; cathepsin D, n-mol/min/ml tyrosine released; acid DNase, n-mol/min/ml acid-soluble nucleotide released; total protein, mg/ml of fraction; radioactivity, ct/min/ml (■-■).

Majority of purified virus always sedimented to the bottom of the tube (Fig. 4f), only about 6% being identified at the lysosomal density. In the presence of ML fraction from uninfected cells most of the virus was found at the bottom of the tube. The amount of recoverable virus was dependent on the multiplicity input within the range 0.1 to 10 ID/cell.

After 24 h infection the ML fraction showed a similar distribution of enzymic activity...
with a peak at 1.18 g/ml, but the amount of the activity was greatly reduced compared to the 1 h infected cells. A smaller amount of virus sedimented with lysosomes. Electron microscopy of the 24 h infected cells revealed no virus particles in the dense granules, even after staining for acid phosphatase. The amount of radioactive virus was proportional to the multiplicity. A significant reduction ($P = 0.01$ to $P = 0.05$) both in total protein content and in acid hydrolases was found in cells infected for 24 h with either 1 or 10 ID/cell of SFV as compared to control cells. No significant change in these parameters occurred after either 1 or 7 h of infection. Lysosomal injury was detectable after 24 h infection.

The technique of Blackman & Bubel (1969) was used to determine the amount of protein released from control and infected cells. Growth medium was removed from confluent cell cultures and replaced by serum free basal Eagle's medium containing 0.2% methocel (methyl cellulose, DOW Chemical). Samples of $10^7$ cells were infected with $10^5$ ID/cell of SFV. Supernatant fluids from the cultures after 1, 7 and 24 h of incubation were centrifuged at 17,000 g for 20 min, and examined for protein content. Control cells released only 19 g/ml of protein over the 24 h period, whereas infected cells released significant amounts of protein both at 7 h (153 g/ml) and at 24 h (225 g/ml). The cells were shown to remain viable under these conditions, and their number remained constant over the 24 h experimental period. Virus multiplication occurred normally in the infected cells, virus being released into the medium after 24 h, and representing about 11% of the total proteins found in the medium. Enzymic assays were performed on the culture media, and acid hydrolases activities were detectable only in supernatant fraction from 24 h infected cells. The increased level of protein in the medium after 24 h infection suggested cellular injury. The release of lysosomal enzymes at the same time as virus production might be responsible of the alteration of the cell membrane.

Acid hydrolases were prepared from the ML fraction of uninfected cells. The ML fraction was disrupted by freezing and thawing, and the resultant suspension was centrifuged at 15,000 g for 30 min to eliminate membrane fragments. The supernatant fraction contained active lysosomal enzymes. Purified SFV ($1.2 \times 10^7$ ID) was incubated with a quantity of this supernatant fraction corresponding to $1.5 \times 10^7$ cells for 2 h at 37 °C. No inactivation of the virus was observed.

Our electron microscopic and density gradient centrifuging data show that lysosomes are involved very early in the infection of primary kidney cell cultures by SFV. At 1 h after infection the majority of the cell-associated virus particles were within the lysosomes; even at 10 min post-infection some interaction has already occurred. The penetration and uncoating of the free virus in the cytoplasm followed a pattern similar to that observed for vaccinia virus (Dales, 1963; Dales & Kajioka, 1964), but took a longer time. It remains to be seen if there is a relationship between the high percentage of SFV particles which pass into lysosomes and the large number of virus particles corresponding to a single infectious dose: 1600 particles for SFV (Jacquemont et al. 1972) as compared with 100 particles for vaccinia virus (Gold & Dales, 1968). No uncoating of SFV was observed in the lysosomes over a 4 h period. In some cases it appeared that the virus envelope disappeared simultaneously with the disintegration of the lysosomal membrane.

Lysosomes appeared to become more fragile towards the end of the virus multiplication cycle, near the time of cellular degeneration. Lysosomal enzymes were released from SFV infected cells after 24 h of infection, at which time the electron microscope showed that the virus particles were no longer within the lysosomes, although the release of proteins was already observed at 7 h. The virus particles themselves are resistant to the free acid hydrolases, and it is suggested that these enzymes might affect the plasma membrane of the cells,
facilitating the release of virus, and of cell proteins. It is possible that the nuclear membrane could also be affected.

The authors are indebted to Professeur R. Sohier for his advice, to Miss J. Auray and Miss M. F. Coquillion for their technical assistance and to Dr T. Greenland for reading the manuscript.

This work was partly supported by the Ligue Nationale Française contre le Cancer, Equipe de Recherche 124 du C.N.R.S.

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(Received 25 May 1973)