Studies on the Lysosomes of L132 Cells Infected with either Rhinovirus Type 2 or Poliovirus Type 1

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SUMMARY

Samples taken from either rhinovirus- or poliovirus-infected suspensions of L132 cells at various times during the growth cycle were assayed for intra- and extracellular virus infectivity, trypan blue uptake and release of acid phosphatase from lysosomes. At similar times infected L132 cell monolayers were observed for cell rounding (virus c.p.e.). At 16 h after infection with rhinovirus 2, cells showed no change in the distribution of acid phosphatase activity but had undergone extreme cytopathogenic changes; at this time 99% of the virus was intracellular and few cells took up trypan blue. Poliovirus-infected cells showed no change in the distribution of acid phosphatase at 6 h after infection when cytopathogenic changes were extreme, but 2 h later when cells began to take up trypan blue and release virus, acid phosphatase was released from the lysosomes.

It is suggested that lysosomal enzymes have no role in the induction of virus c.p.e. but are involved at a later stage of degeneration of the cell.

During an investigation on the effect of rhinovirus infection on lysosomes of L132 cells, we failed to detect any change in the distribution of a lysosomal enzyme even 4 h after the cells were rounded and detached from Petri dishes, indicating extreme c.p.e. This observation conflicts with the suggestion that the release of lysosomal enzymes causes c.p.e. in virus infections (Defendi, 1962; Allison & Sandelin, 1963; Mallucci & Allison, 1965; Guskey, Smith & Wolff, 1970; Mosser et al. 1972), although there are conflicting reports that lysosomal enzymes are not responsible for c.p.e. (Wolff & Bubel, 1964; Walker & Pumper, 1968; Sato, Righthand & Karzon, 1971).

In an attempt to resolve this problem we studied the integrity of lysosomes in suspended L132 cells infected with either rhinovirus 2 or poliovirus 1. In preparing suspensions of lysosomes we avoided low concentrations of sucrose and use of the Dounce homogenizer. Under these conditions, there was a marked release of acid phosphatase from the lysosomes of infected cells at the time of cell death, but at the time of maximum c.p.e. there was no detectable change in the distribution of lysosomal enzymes. This suggested that lysosomal enzymes caused no c.p.e. in the virus-host systems studied.

L123 cells (Davis, 1960) and HeLa cells were grown and propagated in growth medium (GM) or maintenance medium (MM) as previously described (Stott & Heath, 1970). The virus strains used were rhinovirus type 2 (HGP) and poliovirus type 1 (Brunhilde). Rhinovirus 2 was assayed as described by Stott & Heath (1970). Poliovirus type 1 was assayed similarly with the exception that DEAE-dextran was excluded from the overlay medium. The viability of cells was determined by diluting a sample in 0.1% trypan blue in phosphate-buffered saline. Cells which excluded the dye on microscopical examination were taken as viable.

Suspension cultures of L132 cells were maintained at a concentration of 2 to 3 x 10⁶ cells/ml (Stott & Heath, 1970), and infected at an input multiplicity of 100 p.f.u./cell. After adsorp-
tion for 30 min at 37 °C, infected cells were washed three times in MM at 4 °C and incubated at either 33 °C (rhinovirus) or 37 °C (poliovirus). Control cells were mock-infected. Samples were taken at frequent intervals during the growth cycle in order to determine cell viability, virus infectivity and the latency of acid phosphatase. The results below were compiled from three such experiments.

Monolayer cultures of L132 cells were prepared in GM by seeding 2 x 10^6 cells/plate onto 6 mm plastic Petri plates (Nunclon Ltd.) and incubating overnight at 37 °C in an atmosphere of 5% CO_2 in air. The resulting monolayers were inoculated with virus at an input multiplicity of 100 p.f.u./cell: after adsorption for 30 min at 37 °C the monolayers were washed three times in MM at 4 °C and covered with 5 ml MM. Control cells were mock-infected. Plates were gently rocked at either 33 °C (rhinovirus) or 37 °C (poliovirus). At frequent intervals all plates were observed microscopically for c.p.e. and duplicate samples were frozen, thawed and assayed for infectivity. Morphological changes were classified on a + to ++++ scale, indicating that approximately 25%, 50%, 75% and 100% of cells were rounded or detached from the plate. Where cells were detached from the plate they were examined for viability as described above. Observations were based on at least 40 plates per experiment and compiled from three separate experiments.

For determination of lysosomal enzyme activity and latency, samples of 8 x 10^7 suspended cells were cooled to 4 °C and the cells harvested by centrifuging at 5000g for 2.5 min. The supernatant fluids were decanted and the cells washed once with 0.25 M sucrose (Analar-Hopkin & Williams Ltd.) before resuspension in 0.5 ml 0.25 M sucrose. The cells were then disrupted by exposure to pH 11.0 for 2 min at 0 °C (Lee, 1970). During disruption and in all subsequent procedures the concentration of sucrose was maintained at 0.25 M.

Acid phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.2) was assayed in triplicate at pH 4.75, using β-glycerophosphate (Grade I, Sigma Ltd.) as substrate (Allen & Lee, 1972). Sp. act. is expressed in milliunits (mU)/mg protein. The latency of the enzyme is defined as the percentage of the total activity present in the homogenate which was unmasked by incorporation of 0.05% Triton X-100 (Sigma Ltd.) into the reaction mixture. Consequently, the decrease in the latency value indicates the extent to which enzymes have been released from lysosomes.

Poliovirus infection of suspended L132 cells produced maximum yields of virus at 6 h after infection when 99% of the infective particles were intracellular (Fig. 1). Thereafter, although the overall yield remained constant, the proportion of extracellular virus increased to 95% by 12 h after infection. The uptake of trypan blue by poliovirus-infected cells correlated well with virus release. The percentage of cells adsorbing the dye increased slowly between 5 h and 9 h after infection and then rapidly until 95% of cells were stained at 2 h (Fig. 1). The sp. act. of acid phosphatase in poliovirus-infected cells decreased only slightly from 5.2 to 3.8 mU/mg protein during the 12 h period of observation. The proportion of this activity unmasked by Triton X-100 did not differ from that in mock-infected cells for 8 h after infection. Thereafter, it fell sharply indicating the release of enzyme from lysosomes.

Rhinovirus 2 replicated more slowly than poliovirus in suspended L132 cells (Fig. 2). Maximum infectivities of virus were reached 8 h after infection and in the succeeding 8 h decreased only slightly. Throughout this 16 h period 99% of the virus remained intracellular. Rhinovirus infected cells did not begin to adsorb trypan blue until 16 h after infection. The sp. act. of acid phosphatase in rhinovirus-infected cells, and the proportion of this activity unmasked by Triton X-100, remained unchanged throughout the period of observation (Fig. 2).
Virus production and release and the uptake of trypan blue were the same in monolayer cultures and in suspended cells for both poliovirus and rhinovirus infections. The appearance of c.p.e., or cell rounding, was first detected in monolayer cultures 4 h after infection with poliovirus and 6 h after infection with rhinovirus. All cells were rounded at 6 h after infection with poliovirus or 12 h with rhinovirus (Figs. 1, 2).

Our results on the infection of L132 cells by these picornaviruses demonstrate that the sp. act. of acid phosphatase did not increase and that, when it occurred, this enzyme was released from lysosomes several hours after the rounding of the cells or observation of c.p.e. Thus, the labilization of lysosomes or increased activity of their enzymes were not necessary for the production of c.p.e. This was particularly clear during rhinovirus infection when no change in lysosomal enzymes was detected even 4 h after all the cells were rounded (Fig. 2). 

Our findings indicate that the release of lysosomal enzymes in poliovirus infected cells slightly precedes the release of virus and an increase in the proportion of cells adsorbing trypan blue. These effects imply a general breakdown in the integrity of cellular membranes, and may be a consequence of the change in the properties of lysosomes. This would be consistent with the suggestion that the damage in cells subjected to stress becomes irrever-
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possible only when lysosomes are disrupted (Allen & Lee, 1972). Further evidence for the involvement of lysosomal enzymes in cell lysis by picornaviruses is given by Amako & Dales (1967) who showed that the loss of granule-associated lysosomal enzyme activity after infection of L-cells with S or L variants of Mengovirus was related to the time of cell lysis rather than to the synthesis and release of virus which was similar for both variants.

The reasons why our results are at variance with much published work implicating labilization of lysosomes as a cause of c.p.e. require explanation. The different types of viruses and cells used may account for some of the discrepancies but even if comparisons are limited to picornavirus infections of continuous cell lines, most results suggest that enzymes are released from lysosomes much earlier in the virus growth cycle than is indicated by our results. More important causes for these differences are likely to be the definition of c.p.e. and the method used to prepare cell extracts and lysosomes. We have, like Bablanian, Eggers & Tamm (1965), defined c.p.e. as cell rounding. This is the simplest, most readily observable, and probably the earliest c.p.e. of many virus infections. Other workers have either not defined c.p.e. at all (Flanagan, 1966) or have tended to equate it with cell lysis (Blackman & Bubel, 1969; Lake, Winkler & Ludwig, 1970) or with more complex intracellular changes observed by light or electron microscopy (Bienz, Egger & Wolff, 1973). Other workers have prepared lysosomes using physical disruption of cells by Dounce homogenization in hypotonic solutions (Wolff & Bubel, 1964; Flanagan, 1966; Blackman & Bubel, 1969; Guskey et al. 1970; Mosser et al. 1972; Bienz et al. 1973). Our disruption of cells at high pH, unlike Dounce homogenization, ruptured all the cells in the suspension. Consequently, we did not select for the most readily disrupted cells. Furthermore, we were able to use 0.25 M-sucrose during disruption so that the lysosomes were not exposed to hypotonic solutions in which they are unstable (Applemans & de Duve, 1955). It is possible that, during the early stages of virus infection, lysosomes enlarge and become more vulnerable to damage by Dounce homogenization. Results obtained by this method would then indicate the early release of lysosomal enzymes, although there would be no release in intact cells. Thus, damage by Dounce homogenization may explain why many workers describe the release of lysosomal enzymes as early as 4 h after poliovirus infection whereas our results indicate no such release until at least 8 h after infection.

It has been shown by electron microscopy (Dales et al. 1965; Skinner, Halperen & Harkin, 1968) and by incorporation of choline (Penman, 1965) that during replication many picornaviruses cause a proliferation of cellular membranes. Skinner et al. (1968) showed with echovirus 12, that the increase in intracellular membranes from 5 h after infection coincided with the appearance of cytopathogenic effects and that both were inhibited by 2-(α-hydroxybenzyl)-benzimidazole. It is possible, therefore, that the cell rounding or cytopathogenic effects, so characteristic of infection by picorna – and other viruses, may be due in part to a redistribution of cell membrane components. Rounding of the cell reduces the ratio of surface area to vol. and thus the cell may increase the proportion of inner membrane or endoplasmic reticulum upon which occur the synthesis of virus RNA and protein (Caliguiri & Tamm, 1970). Although there is little firm evidence for this hypothesis, our results indicate that the earliest virus-induced cytopathogenic effects are not mediated through lysosomal enzymes, and are consistent with the suggestion of Lake & Ludwig (1971) that a breakdown of normal cellular membranes is the primary result of virus infection.
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