ACTIVATION OF LYSOSOMAL ENZYMES IN VIRUS-INFECTED CELLS AND ITS POSSIBLE RELATIONSHIP TO CYTOPATHIC EFFECTS

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One type of cell-virus interaction leads to more or less severe cytopathic effects, which may take various forms (1). However, such cytopathic effects are not always observed when virus multiplication occurs, *e.g.* with influenza A in human embryo lung cultures (2). Cases are also known in which severe cytopathic effects take place but there is no multiplication of virus, *e.g.* vaccinia in macrophage cultures (3). The same virus may be cytopathic when multiplying in one cell system but not when multiplying in another, *e.g.* ECHO 28 (2060-JH) which is cytopathic in monkey kidney cells but not in HEp2 cells (4). And when multiplying in the same cell system one virus may be cytopathic and another not, *e.g.* influenza B but not influenza A in human embryo lung cells (2). Furthermore, Vainio and his colleagues (5) have shown that in the presence of antihistamines multiplication of mouse hepatitis virus is not inhibited but cell degeneration is markedly reduced.

All these observations show that severe cell damage is not a necessary consequence of virus multiplication, but may be due to a process which is sometimes set in motion during virus multiplication and sometimes not. In looking for such a mechanism our attention was directed to the work of de Duve (6, 7), Novikoff (8), and others on lysosomal enzymes, activation of which is believed to play an important part in cell damage following exposure to a variety of noxious agents. These enzymes, including acid phosphatase, acid ribonuclease, acid deoxyribonuclease, acid protease, and β-glucuronidase, are normally present in a group of cell particles known as lysosomes, which present a variety of appearances in electron micrographs (8, 9). In conventional cell fractionation procedures, most activity of the lysosomal enzymes is recovered in the mitochondrial fraction, with less in the supernatant (6). During activation the enzymes are released from the lysosomes into the surrounding cytoplasm. In fractionated cells the total activity of lysosomal enzymes is then increased, and the majority of activity is found in the supernatant fraction (6, 7). It can readily be appreciated that excessive release of such hydrolytic enzymes into the cytoplasm could be damaging to cells.

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We have investigated two systems, mouse liver cells infected with mouse hepatitis virus (MHV3) *in vivo* and monkey kidney cells infected with vaccinia virus *in vitro*. In both cases activation of lysosomal enzymes has been found by conventional techniques (6, 7). The results show that activation of lysosomal enzymes is associated with the appearance of cytopathic effects, and the possibility that this relationship may be causal is discussed. These results are complementary to histochemical studies of virus-infected cells described elsewhere (10).

Material and Methods

Mice.—Male mice of strain VSBS/NIMR, weighing 10 to 12 gm, were used.

Monkey Kidney Cells.—Rhesus monkey kidney cells were kindly supplied by the Biological Standards Division of this Institute, Hampstead, and cultivated in flat bottles for 6 to 7 days in the following medium: Hanks' saline, 88 parts; lactalbumin hydrolysate, 5 parts; calf serum, 5 parts; 4.4 per cent $NaHCO_3$, 1 part; streptomycin, 100 μg per ml; penicillin, 100 units per ml. On the 7th day the medium was changed to Parker's solution 199, 90 parts; calf serum, 10 parts. In 8 to 10 days, when the monolayer was confluent, the cells in some bottles were infected while others were kept as controls.

Viruses.—Mouse hepatitis virus, MHV3 (11), was obtained from Dr. A. Gledhill and propagated and titrated by intraperitoneal injection in weanling mice. Vaccinia virus: the Lister Institute egg-adapted strain of vaccinia virus, propagated and titrated in the chorioallantoic membranes of 11-day-old chick embryos, was used. Mice were infected intraperitoneally with approximately 10^5 LD_{50} of MHV3 and monkey kidney cells with approximately 10 pock-forming units per cell.

Preparation of Homogenates.—Mouse livers were chilled immediately after death and weighed. A 5.5 per cent suspension (wet weight/volume) in ice cold 0.25 M sucrose was homogenized under standard conditions in a glass tube fitted with a teflon cylinder rotating at 2000 RPM. with 0.025 inches clearance. The homogenate was centrifuged at 2000 *g* in the cold for 15 minutes, and the deposit (nuclei and cell debris) discarded. The supernatant was centrifuged for 1 hour at 12,000 *g* in a refrigerated centrifuge and the supernatant and resuspended deposit (particulate, that is, mitochondrial + lysosomal) fractions used for enzyme assay.

Pools of 4 to 6 bottles containing infected and control monolayers of monkey kidney cells were used for each experiment. For effective homogenization it was found necessary to allow the cells to swell in hypotonic solution. They were recovered in Gey's solution diluted with an equal volume of distilled water, and homogenized in 0.125 M sucrose. Otherwise the procedure was the same as for liver cells.

Enzyme Assays.—

β-glucuronidase: 0.5 ml of 0.1 M sodium acetate buffer, pH 5.0; 0.1 ml of 0.01 M phenolphthalein—β-glucuronide and 0.1 ml enzyme preparation were incubated for 20 minutes at 37°C. The reaction was stopped by adding 2 ml of 0.4 M glycine, the pH of which was adjusted to 10.7 with N NaOH. After centrifugation for 10 minutes at 2000 *g* the samples were diluted with an equal volume of distilled water. The amount of phenolphthalein liberated was measured in a Unicam S.P. 500 spectrophotometer at 545 mμ.

Acid phosphatase: 2.0 ml of 0.1 M sodium acetate buffer, pH 5.0, 0.2 ml of 0.13 M sodium—β-glycerophosphate, and 0.2 ml enzyme preparation were incubated for 20 minutes at 37°C. The reaction was stopped by adding 0.8 ml of 8 per cent perchloric acid and the samples centrifuged for 10 minutes at 2000 *g*. Inorganic phosphate was estimated in the supernatant by the technique of Lowry and Lopez (12), reading the optical density at 650 mμ 10 minutes after addition of molybdate.

Acid protease, DNAase and RNAase: These were assayed as follows: 0.5 ml of 0.1 M sodium acetate buffer, pH 5.0, 0.05 ml of substrate, and 0.2 ml enzyme preparation were incubated at 37°C. for 1 hour. The following were used as substrates: 1 per cent bovine homoglobin (Armour), 1 per cent yeast RNA, prepared by the technique of Kay and Dounce (13) and 0.5 per cent calf thymus DNA, prepared by the technique of Kay *et al.* (14). The reaction was stopped by adding 0.7 ml uranyl acetate—perchloric acid (15). The samples were centrifuged for 15 minutes at 2000 *g* and the supernatant diluted 1 in 2 with distilled water before reading optical densities at 280 mμ and 260 mμ in a Unicam S.P. 500 spectrophotometer. Results are expressed in optical density units so recorded.

The concentrations of protein in enzyme preparations were also determined by the biuret technique (16) and results expressed in terms of protein concentration showed essentially the same changes as those described below.

RESULTS

Similar results were obtained in several experiments, some of which are summarized in Tables I and II and Figs. 1 and 2. In keeping with the reports of de Duve (6) and others, most activity of lysosomal enzymes in normal liver

TABLE I

Enzyme Activities in the Lysosomal (L) and Supernatant (S) Fractions of Mouse Livers before and after Inoculation with MHV 3

Activities are expressed in optical density units as described in the text.

Days after infection	β-glucuronidase		Acid-phosphatase		Acid protease		Acid RNAase		Acid DNAase	
	L	S	L	S	L	S	L	S	L	S
Uninfected	0.12	0.08	0.46	0.28	0.08	0.04	0.69	0.45	0.08	0.03
1	0.09	0.16	0.34	0.38	0.07	0.08	0.64	0.28	0.09	0.02
2	0.17	0.14	0.42	0.44	0.05	0.09	0.68	0.92	0.08	0.08
3	0.08	0.20	0.36	0.59	0.01	0.11	0.88	1.04	0.09	0.13
4	0.07	0.38	0.39	0.61	0.01	0.17	0.76	1.11	0.08	0.17

TABLE II

Enzyme Activities in the Lysosomal (L) and Supernatant (S) Fractions of Monkey Kidney Cells before and after Infection with Vaccinia Virus

Activities are presented in optical density units as described in the text.

Hours after infection	β-glucuronidase		Acid phosphatase		Acid-protease		Acid RNAase		Acid DNAase	
	L	S	L	S	L	S	L	S	L	S
Uninfected	0.19	0.11	0.19	0.08	0.10	0.04	0.64	0.56	0.03	0.03
6	0.15	0.30	0.15	0.19	0.05	0.06	0.61	0.59	0.05	0.06
12	0.27	0.32	0.10	0.25	0.05	0.07	0.68	1.16	0.07	0.13
16	0.04	0.25	0.27	0.40	0.09	0.12	0.60	1.32	0.04	0.15

cells was present in the particulate fraction, with less in the supernatant. The same was true of monkey kidney cells in culture.

After infection with mouse hepatitis virus and vaccinia virus there was a progressive increase in total activity of lysosomal enzymes, which was practically confined to the supernatant fraction. In the case of some enzymes, *e.g.*

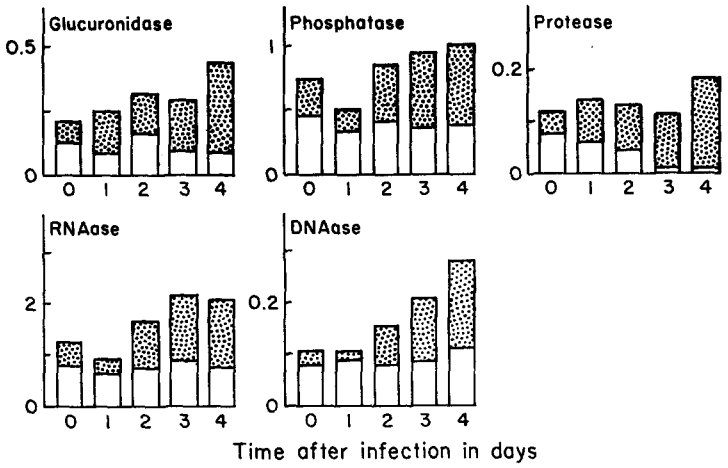

FIG. 1. Enzyme activities in lysosomal and supernatant (dotted areas) fractions of mouse iver at various times after infection with MHV3.

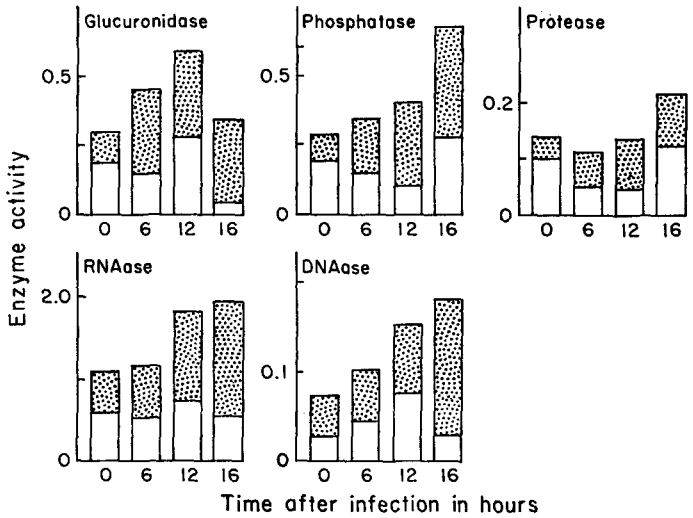

FIG. 2. Enzyme activities in lysosomal and supernatant (dotted areas) fractions of monkey kidney cells at various times after infection with vaccinia virus.

protease, activity in the particulate fraction decreased. In the liver cells increased activity of lysosomal enzymes was demonstrable on the 2nd day after infection—before parenchymal cell damage was visible histologically. In the vaccinia virus–infected monkey kidney cells, changes in enzyme activity were demonstrable 6 hours after infection—again before cytopathic effects were observed on microscopical examination.

DISCUSSION

Previous workers have reported an increase in liver arginase and plasma fumarase in mice infected with MHV3 (17), and decreased isocitric, succinic, and lactic dehydrogenase, and increased glucose-6-phosphate dehydrogenase activities, demonstrated histochemically, in livers of mice infected with MHVS (18). The experiments described in this paper show that the growth of two viruses in host cell systems, one *in vivo* and one *in vitro*, is accompanied by activation of lysosomal enzymes. The activation is manifested in most cases by an increase in total enzyme activity and in all by an increase in enzyme activity in the supernatant fraction. The latter reflects escape of enzymes from cell organelles (lysosomes) into the cytoplasm (6, 7). The question then arises whether the activation of lysosomal enzymes in virus-infected cells precedes cell damage and contributes to this process, or whether the enzymes are activated in cells the metabolism of which is already severely disturbed and which are in any case moribund. This question is difficult to answer directly, but several points favour the former interpretation.

The first point is that the results here described are not an artefact of homogenization. Allison and Burstone (10) have found that in sections of livers of mice infected with MHV3 and examined histochemically there is a marked focal activation of acid phosphatase, which is diffuse and not particulate, as in normal cells. In recent unpublished experiments using histochemical techniques designed for analysis of lysosomes (19), marked activation of lysosomal enzymes was visible in livers of mice 48 hours after infection with MHV3.

This is the second point: that activation of lysosomal enzymes, as demonstrated both biochemically and histochemically, precedes visible cell damage; however, it must be recognized that histological change is a rather insensitive indicator of incipient cell degeneration. The third point is that virus multiplication can occur in the absence of marked cytopathic effects. This suggests that the metabolic changes associated with virus multiplication (in particular, synthesis of viral nucleic acid and protein) are not necessarily damaging to cells. Hence the analogy which is sometimes quoted with unbalanced or lethal synthesis in thymineless strains of bacteria or bacteria grown in the presence of 5′-fluorodeoxyuridine (*Cf.* reference 20) does not seem to be close. In any case, there is evidence that in thymineless strains of bacteria abnormal DNA with single chain scission is present, possibly owing to nuclease activity (21). Marked

activation of DNAase and RNAase in bacteria shortly after phage infection has been recognized for a number of years (20). In this connection Baltimore and Franklin (22) have recently reported inhibition of nuclear RNA synthesis in L cells infected with mengovirus. They suggest that this will interfere with protein synthesis and lead to eventual cell death. They recognize, however, that this cannot be the complete explanation of viral cytopathogenesis since actinomycin D–treated cells, in which nuclear RNA synthesis is inhibited to the same degree, show much less drastic morphological changes.

Lysosomes might conceivably participate in three stages in the life cycle of animal viruses: during penetration of the virus into the host cell, during virus growth and during virus release. Evidence is accumulating that animal viruses are taken into cells by a process akin to pinocytosis and appear to disintegrate within pinocytotic vacuoles (*Cf.* reference 23). In other situations in which pinocytosis or phagocytosis occurs, including uptake of bacteria by mammalian leucocytes, there is activation of lysosomes around the pinocytotic vacuoles (8, 24) and the same may occur during penetration of animal viruses. Possibly lysosomal enzymes participate in the "uncoating" mechanism by which virus nucleic acid is released, in which case the nucleic acid would have to emerge in a form resistant to the action of lysosomal nucleases.

Lysosomal enzymes might also be involved in the breakdown of host cell polynucleotides which results in a markedly increased pool of acid-soluble nucleotides in infected cells (25). In the same cell-virus system, HeLa cells infected with herpes simplex, Dr. A. Newton (private communication) has found an increase in acid DNAase reaching a maximum 6 hours after infection. Hanafusa (26) has reported increased DNAase activity in vaccinia-infected L cells, but has not characterized the enzyme as lysosomal. Kovacs *et al.* (27) have reported an early increase in acid RNAase after infection with poliovirus in most preparations of HeLa cells studied. They also reported an abrupt rise in diffusible acid phosphatase in supernatant of infected cell cultures; in the same supernatants glucose-6-phosphatase was only slightly increased, and it was concluded that only the lysosomal enzyme is liberated 3 to 6 hours after infection. Activity of phosphatase, and another lysosomal enzyme, phosphoprotein phosphatase (28) might contribute to the increased intracellular pool of phosphate and phosphate acceptor, and concomitant enhancement of glycolysis, which is a common feature of virus-infected cells (*Cf.* reference 20)

Thirdly, lysosomal enzymes might play a part in cell degeneration, which facilitates the liberation of viruses the synthesis of which is not completed at or near the cell surface, *e.g.* adenoviruses, which are packed in nuclei. It also seems likely that some of the changes in virus-infected cells shown in published electron micrographs are due to enlargement and alteration in the structure of lysosomes, and damaging effects of lysosomal enzymes on mitochondria. Examples are the myelin figures and large bodies observed by Svoboda *et al.* (29) in livers of mice infected with MHV3.

Why lysosomal enzymes should sometimes be activated in virus-infected cells is unknown, but certain metabolic changes may favour this process. Thus, quite brief periods of anoxia are sufficient to cause activation of liver cell lysosomal enzymes (6); the lysosomes are also highly sensitive to acidic conditions (6), and possibly a fall in intracellular pH follows viral infection. Agents such as benadryl which limit cell damage after mouse hepatitis virus infection (5) also reduce damage due to administration of carbon tetrachloride and other noxious agents, in which release of lysosomal enzymes is believed to play a part (6). Conceivably agents such as benadryl act by stabilizing lysosomal and other intracellular membranes. According to the view here advanced, degeneration of cells after virus infection is a process similar to that induced by other agents. The fact that a wide range of viruses can produce such effects suggests that a common mechanism may be responsible and that it is unnecessary to postulate the existence of virus-specific enzymes to explain the observed metabolic and morphological changes in infected cells.

SUMMARY

Activities of the enzymes β-glucuronidase, acid phosphatase, acid DNAase, acid RNAase, and acid protease have been measured in the lysosomal and supernatant fractions of mouse liver cells and monkey kidney cells before and after infection with mouse hepatitis virus and vaccinia virus, respectively.

In the infected cells there was easily measurable release of lysosomal enzymes into the supernatant fraction. Evidence was presented that this is not an artefact of homogenization and precedes cell degeneration demonstrable histologically.

It is suggested that release of lysosomal enzymes may explain some of the biochemical changes found in infected cells and may contribute to the cytopathic effects of some viruses.

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