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Liver Autophagy in the Influenza B Virus Model of Reye's Syndrome in Mice

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Biochemical evidence is presented for the autophagic destruction of liver mitochondria in the influenza B virus model of Reye's syndrome in mice. Separation of lysosomes and autophagic vacuoles from mitochondria was accomplished by prior treatment of the mice with Triton WR-1339, resulting in uptake of detergent by these organelles (tritosomes), reducing their densities. The organelles were banded in a discontinuous sucrose gradient. Total protein in the heavy tritosomal fraction increased from 1-2% in controls to 7-8% in virus-treated animals. Ornithine carbamoyl transferase (OCTase), a mitochondrial marker, increased from 2–3% (controls) to 11–15% (virus-treated), and glucose-6-phosphatase, a marker for endoplasmic reticulum, increased from 1–2% (controls) to 8–10% (virus-treated). β-Galactosidase, a soluble enzyme in the lysosome, and OCTase also increase in the cell extract fraction following virus treatment, indicating that there was turnover of heavy lysosomal contents.

Key words: Reye's syndrome, liver autophagy, influenza B virus, ornithine carbamoyl transferase, glucose-6-phosphatase, tritosomes

Davis and coworkers have recently reported the development of an experimental mouse model for Reye's syndrome [1]. A striking feature of the electron micrographs of liver in this report was the arrangement of rough endoplasmic reticulum around the damaged mitochondria. This has been shown to be characteristic of an autophagic process [2–4]. Similar figures can be seen in electron micrographs of biopsy/autopsy liver specimens from patients with Reye's syndrome [5,6] and in electron micrographs in the animal model described by Hug et al [7]. Double, and in some cases, triple membranes can be discerned surrounding the distorted mitochondria. We report here biochemical evidence supporting the morphological evidence that autophagy is induced in the liver in this model.

The autophagic process produces a population of lysosomes containing the intracellular material being turned over. To separate the lysosomes from the mitochondria, both of which sediment to an average specific gravity of 1.2, we employed

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the method of Leighton et al for the preparation of "tritosomes" [8]. Uptake of the detergent Triton WR-1339 by liver lysosomes causes the lysosomes to band at a lower density than do mitochondria in a discontinuous sucrose gradient.

MATERIALS AND METHODS

Weanling male Balb-C mice (Harlan Industries), weighing 10 to 12 g, were injected intraperitoneally with 5% Triton WR-1339 (Ruger Co.) (0.9 mg/g body weight). Two and a half days later, experimental animals were injected intravenously with 0.5 ml (15,000–20,000 HAU) of influenza B/Lee/40 virus grown in eggs [1]. Control animals were injected with phosphate-buffered saline. The mice were killed 22 to 24 hr later by exsanguination and the livers rapidly removed to ice.

Typically, three livers were combined for fractionation. The livers were homogenized for 1 min in three volumes of cold 0.25 M sucrose, 0.005 M Tris-Cl, pH 8.0, and centrifuged for 10 min at 600g. An aliquot of each homogenate was retained for initial enzyme assays and protein determinations. The pellets were resuspended and recentrifuged twice. The final pellets were suspended in two volumes and reserved as the nuclear fraction (N). This fraction presumably contained some cell debris and unbroken cells in addition to nuclei.

The combined supernatants were centrifuged for 15 min at 13,600g. The loose pink layer of sediment (microsomal fraction) was removed with the supernatant by aspiration. The pellets were resuspended and recentrifuged twice. The final pellets were suspended in cold 1.5 M sucrose (ρ = 1.20)(M/L). The supernatant was centrifuged for 30 min at 35,000g. These pellets, representing the microsomal fraction, were suspended in two volumes 0.7 M sucrose (P). The supernatants were retained as the cell extract (CE).

The (M/L) pellet suspensions were placed in centrifuge tubes. An equal volume of cold 1.0 M sucrose (ρ = 1.13) was layered on each, followed by a ½ volume of 0.37 M sucrose (ρ = 1.05). These were centrifuged for 2.5 hr at 64,000g (22,000 rpm, SW27 rotor, Beckman L-8 preparative ultracentrifuge). Diagrams of the separations in the discontinuous gradients are shown in Figure 1. Sections of each gradient were removed by aspiration. The tritosome fraction (T) included the upper layer and the visible particulate matter at the interface of the ρ = 1.05 and ρ = 1.13 layers. The heavier lysosome (L) fraction included all the rest of the supernatant. In the controls, there was little or no visible material remaining in the supernatant. The mitochondrial pellets (M) were suspended in 2 volumes of 0.7 M sucrose.

The fractions were assayed for enzyme activities: (a) ornithine carbamoyl transferase (OCTase), a mitochondrial enzyme that has been shown to be displaced to the cytosol in the mouse model [9]; (b) aryl sulfatase and (c) β-galactosidase, lysosomal marker enzymes; (d) β-glucuronidase, an enzyme found in both lysosomes and the microsomal fraction; (e) glucose-6-phosphatase (G-6-Pase), normally found only in the microsomal fraction. OCTase was assayed as previously described [9]. All other enzymes were assayed as described by Tulsiani et al [10]. Protein was determined by the method of Miller [12].

RESULTS

The most marked changes in distribution of enzyme activity occurred for OCTase and G-6-Pase. The data from a typical experiment are presented as deDuve

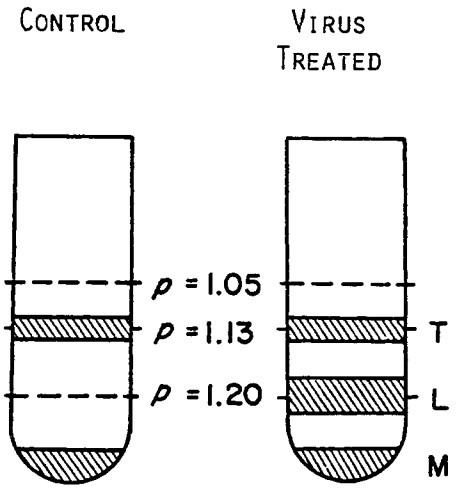

Fig. 1. Discontinuous gradient used for separation of "tritosomes" from mitochondria. Livers from control mice did not yield a visible band at the interface of the two more dense layers. T, triton-containing lysosomal fraction; L, heavier lysosomes; M, mitochondria.

diagrams [11] in Figure 2a and b. For comparison, the deDuve diagram for β-galactosidase is shown in Figure 2c. The area covered by each bar is proportional to the amount of enzyme activity in that fraction. In four experiments, the amount of total protein recovered in the (L) fraction reproducibly increased from 1–2% in controls to 7–8% in virus-treated animals (Table I). Both OCTase, a mitochondrial enzyme, and G-6-Pase, a microsomal enzyme, are also markedly elevated in this fraction. This is consistent with the presence of a visible band only with the virus-treated animals. β-Galactosidase is distributed between the two lysosomal fractions and as a contaminant in the mitochondrial fraction, since not all lysosomes have taken up detergent. Both OCTase and β-galactosidase appear in the cell extract following virus treatment. The distributions of the other enzymes were not altered by virus treatment (data not shown).

DISCUSSION

OCTase

Although the relative specific activity in the cell extract does not increase dramatically with virus treatment, the amount recovered in this fraction was markedly increased. The reduction in amount in mitchondria is marked, and there is an appreciably larger amount of OCTase activity associated with the lysosomal fraction, suggesting that mitochondrial contents are contained in this fraction. OCTase in the cell extract indicates release of autophagic products.

G-6-Pase

This is an enzyme of the endoplasmic reticulum, a component of the microsomal fraction that is the source of the membrane for autophagic vesicles. In the controls, it is found as a slight contaminant in the mitochondrial and tritosomal fractions and to neglible extent elsewhere. (In the differential centrifugation procedure, part of the

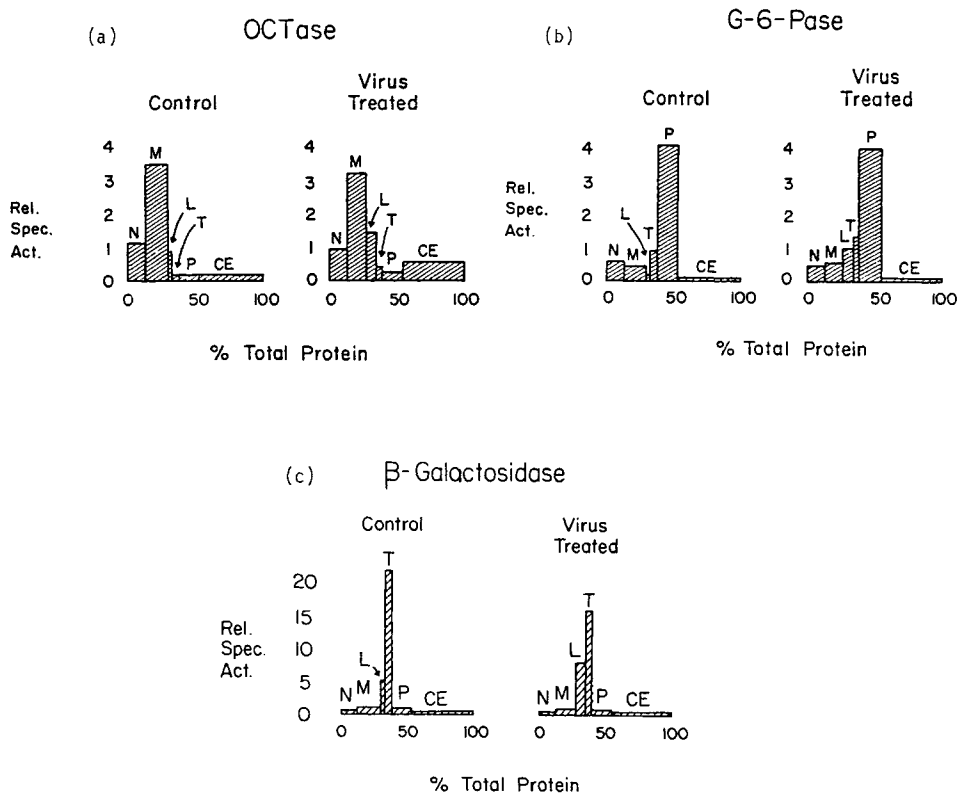

Fig. 2. deDuve diagrams showing enzyme distributions in liver fractions. a) OCTase. b) G-6-Pase. c) β-galactosidase. N, nuclei and cell debris; M,L,T, see legend to Figure 1; P, microsomal fraction; CE, C, postmicrosomal supernatant.

microsomal fraction layered on top of (M/L) and was resuspended during the aspiration of supernatant.) In the virus-treated animals, a significant amount of G-6-Pase is found in the lysosomal fraction, indicating that at least a portion of this population of lysosomes originated from the endoplasmic reticulum. The appearance of G-6-Pase in the lysosomal fraction is used as biochemical evidence for autophagy [3,4].

β-Galactosidase

Some 20% of β-galactosidase is found in the microsomal fraction; 75% is found in the fractions containing lysosomes. Following virus treatment, there is a 3–4-fold increase in the cell extract, suggesting release of autophagic products.

That mitochondrial damage occurs in the liver in Reye's syndrome and in the experimental mouse model for Reye's syndrome has been demonstrated morphologically [1,5,6] and biochemically [1,7,9]. The morphological data also suggest an autophagic process, which we now confirm biochemically in the model. This autophagy may be the primary cause of mitochondrial damage, induced in some manner by treatment with influenza virus, or the natural reaction of the liver cell to turn over damaged organelles, the damage having been caused from some other virus-induced mechanism.

TABLE I. Protein and Enzyme Activities of Subcellular Fractions

	Fraction	Recovered protein[a]	Recovered OCTase activity[a]	Recovered G-6-pase activity[a]	Recovered β-galactosidase activity[a]
Control mice	N	12–15	11–16	7–10	7–11
	M	12–16	64–70	8–10	19–24
	L	1–2	2–3	1–2	16–26
	T	6–8	1–2	5–7	11–24
	P	13–18	4–6	66–73	24–28
	CE	45–51	11–12	5–6	4–6
$\frac{\text{Amount recovered}}{\text{Amount in homogenate}}$		83–87	71–76	100–109	74–78
Virus-treated mice	N	13–15	11–16	5–8	8–11
	M	12–15	42–46	7–9	10–13
	L	7–8	11–15	8–10	19–26
	T	3–4	1–2	5–7	17–24
	P	14–16	3–5	63–67	17–20
	CE	44–48	23–26	4–10	16–19
$\frac{\text{Amount recovered}}{\text{Amount in homogenate}}$		87–92	73–78	103–111	76–83

[a]Percent of total recovered found in each fraction. The ranges shown represent the results of four individual experiments.

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