Biochemical Events in Murine Cytomegalovirus-Infected Mouse Liver (37068)

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Viral hepatitis in the mouse can be readily produced by intraperitoneal injection of murine cytomegalovirus (MCMV). Following MCMV inoculation in weanling mice, there is an increase in virus titer on day one after inoculation (1). The intranuclear inclusions formed as a result of this infection were demonstrated by Ruebner *et al.* (2) in an electron microscope study of weanling mice. Grand and Grandel (3) at light microscope level, examined histochemically these intranuclear bodies in livers of young mice.

The purpose of this investigation was to determine whether alterations in acid hydrolytic enzymes and lipids may occur in liver homogenates of mice during MCMV infection.

Materials and Methods. Mouse cytomegalovirus (MCMV), Smith strain, also termed murine salivary gland virus, was maintained in young (15–20 g) female CFW mice by subcutaneous injection of a suspension of MCMV-infected submandibular glands every two to three weeks. The virus-infected tissue suspensions were prepared by grinding mouse submandibular glands in Hanks' balanced salt solution (BSS) in a ratio of one gland per 1 ml of BSS in a Tenbroek glass grinder. This tissue suspension was centrifuged at 1000 rpm for 5 min. Tissue homogenate was prepared fresh for each experimental series and 0.1 ml was injected ip in each mouse. This ip injection is lethal and results in viral hepatitis as early as the second day. This group was designated as the experimental infected group.

Sham controls. Animals in this group received 0.1 ml ip injection of MCMV-infected submandibular gland homogenate heated in water bath at 65–70° for 30 min to make the virus noninfective. Such a preparation showed no histopathologic evidence and the animals appeared completely healthy.

Normal controls. This group was comprised of young female CFW mice of the same age as the first two groups. In our experience we have never observed any histologic evidence of MCMV carrier state in hundreds of normal mice which we have examined. Furthermore, their submandibular gland homogenates, when taken through three blind transfer passages in normal mice, were consistently histopathologically negative for MCMV infection.

Four animals from each of the three groups were selected randomly and killed by a blow on the head. Their liver lobes, without gall bladders, were removed and chilled immediately in cold 0.25 *M* sucrose solution for five minutes. For enzyme studies the tissues were homogenized and subfractionated the same day. For lipid studies the tissue samples were stored at —20°, but never for more than a week.

Preparation of tissue homogenates and fractionation (for enzyme studies). For enzyme assays the chilled tissues were blotted lightly with paper towels, minced with scissors and homogenized in 9 volumes of 0.25 *M* ice cold sucrose solution by three up-and-down motions of a TRI-R homogenizer (TRI-R Instruments, New York) fitted with a teflon pestle. One portion of the homogenate was immediately centrifuged at 25,000*g* for 25 min at 4°, and assays of "free" enzyme activity were carried out on the supernatant thus obtained. "Bound" enzyme activity was assayed after the resuspended pellet containing subcellular organelles had been exposed to hypo-osmotic treatment by diluting 1:10 with glass-distilled water (to release the membrane bound enzymes). The

sum of "free" and "bound" activities was taken as the "total" enzyme activity.

Measurement of enyme activities. Acid phosphatase was assayed by the method of Torriani (4) at 37°, pH 5.2 using *p*-nitrophenylphosphate as substrate. The liberated *p*-nitrophenol was measured at 400 nm.

Proteolytic activity was determined by incubating 0.5 ml of the tissue homogenate in 1.0 ml of Na-formate buffer pH 3.2 and 0.25 ml of 8% hemoglobin solution at 45° for 30 min. The reaction was stopped by the addition of 5.0 ml of 3% trichloroacetic acid. The optical density of the clear filtrate was measured at 280 nm.

Lipid analysis. Livers removed from the animals were either used freshly or stored for not more than seven days at —20° under nitrogen. Total lipids were extracted with chloroform-methanol (2:1) by the method of Folch, Lees, and Stanley (5). Total cholesterol was determined by the Lieberman–Buchard reaction as specified by MacIntyre and Ralston (6) using a ferric chloride–sulfuric acid color reagent. Phospholipids were assayed by the method of Bartlett (7). Total lipids were estimated by the method of Bragdon (8) and expressed as milligrams of potassium dichromate reduced.

TABLE I. Total Lipids in Liver Homogenates of Control and Virus Infected Mice.[a]

Days after injection	Sham	Virus
4	14.03 ± 0.31[b]	13.41 ± 0.16
2	14.17 ± 0.49	23.63 ± 1.05
3	14.30 ± 0.42	36.32 ± 1.88
4	14.40 ± 0.50	39.56 ± 1.48

[a] Average of four animals ± S.E.M.

[b] Amount of mg of total lipids per g, expressed as mgs of K2 Cr2 07 oxidized/g of liver.

No differences were observed between the sham and normal (not injected) controls with respect to lipids and enzyme behavior. Therefore, only the results obtained on the sham controls will be compared with the experimental results.

Results. Lipids. Total lipids and cholesterol (Tables I and II) in sham control animals stayed constant over the period of four days.

TABLE II. Total Cholesterol in Liver Homogenates of Control and Virus Infected Mice.[a]

Days after injection	Sham	Virus
1	3.68 ± 0.14[b]	3.69 ± 0.16
2	3.64 ± 0.06	5.38 ± 0.16
3	3.97 ± 0.11	6.97 ± 0.22
4	3.40 ± 0.16	7.22 ± 0.28

[a] Average of four animals ± S.E.M.

[b] mg of total cholesterol/g of liver.

On day one after the virus injection, the experimental values were close to those of the controls. Subsequently, the total lipids of the experimental animals showed a steady increase, rising to three times the initial value in four days. Cholesterol also was found to show a steady increase each day resulting in 100% increase in four days over the values on day one. Phospholipids (Table III) stayed constant in both sham and virus-infected animals.

Hydrolytic enzymes. The "free" activity of acid phosphatase (Fig. 1), when expressed as percent of total, *i.e.*, ["Free"/("Free" + "Bound")] × 100 in sham control animals varied between 17–20%, which is within reasonable limits of variation between different animals killed at different days. In virus-infected animals the percentile of activity was higher than in the controls on all post-injection days, but showed a fluctuating or unsteady course.

In Fig. 2, absolute values (*i.e.*, O.D. of the enzyme:substrate reaction product) of free and bound activities are given. It will be noted that in absolute terms free activity remained constant in experimental animals with no significant difference between experimental and control groups. Bound activity was

TABLE III. Total Phospholipids in Liver Homogenates of Control and Virus-Infected Mice.[a]

Days after injection	Sham	Virus
1	4.72 ± 0.31[b]	4.49 ± 0.20
2	4.68 ± 0.28	4.44 ± 0.36
3	5.02 ± 0.16	4.56 ± 0.21
4	5.02 ± 0.16	4.00 ± 0.12

[a] Average of four animals ± S.E.M.

[b] mg of phospholipids/g of liver.

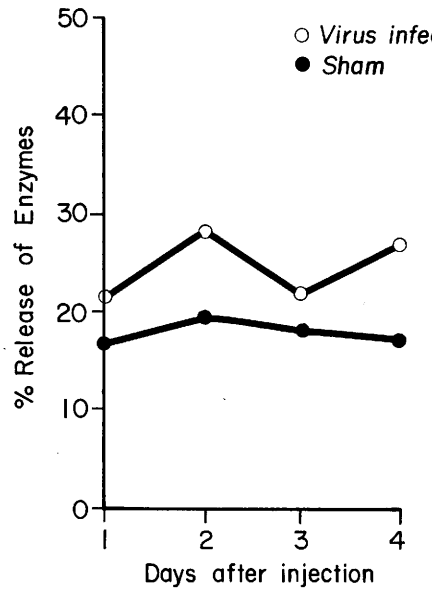

FIG. 1. Activity of free acid phosphatase in liver homogenates of virus-infected and sham-control animals expressed as Free/(Free + Bound) × 100.

lower in virus-infected animals on days 1, 2, and 4, which resulted in higher free activity when expressed as percentage of the total.

Figure 3 depicts the free activity percentages of acid proteinase. Free activity of this enzyme varied between 3.3% to 6.5% in sham control mice sacrificed on four different days after injection. In virus-infected animals the activity was 12.5% on day one, increasing to 19.5% on the second day. It registered a slight dip on the third day, but increased 20.5% on the final day. The difference between percentage of free activity in acid proteinase of liver homogenate from sham and virus-infected animals is quite appreciable as compared with the difference noticed in the activity of acid phosphatase.

Figure 4 shows the absolute values of free and bound activity of acid proteinase. In contrast to the free activity of acid phosphatase which remained unaffected, the absolute activity of free proteinase increased sharply in the experimental animals and was more than three times higher than in the sham control group. The total (free and bound) activity was higher, though only slightly in virus-infected animals on day 2, 3, and 4.

Discussion. Two lysosomal enzymes, *i.e.*, acid phosphatase and acid proteinase were assayed. There was no significant difference in the amount of free acid phosphatase present in the supernatant obtained by spinning down the homogenates of livers from sham and virus-infected animals (Fig. 2). The reason for documenting this as percentage of free activity (Fig. 1) is to show the pitfall in this mode of expression. Free activity when expressed as percentage of total (free + bound) does not convey the true situation. The decrease in bound activity could possibly be due to the result of virus reaction on membrane-bound acid phosphatase which

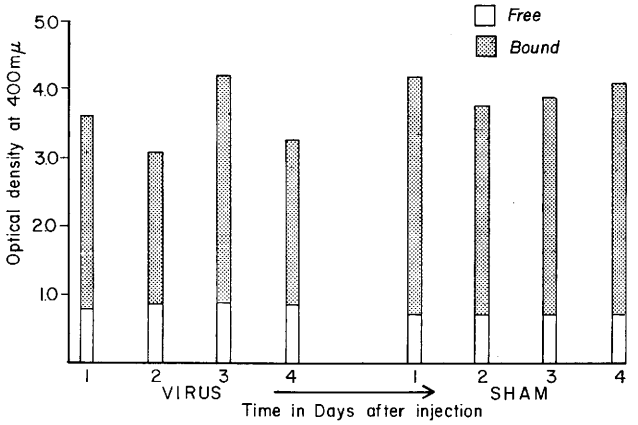

FIG. 2. O.D. of the reaction product of free and bound acid phosphatase from virus-infected and sham-control animals.

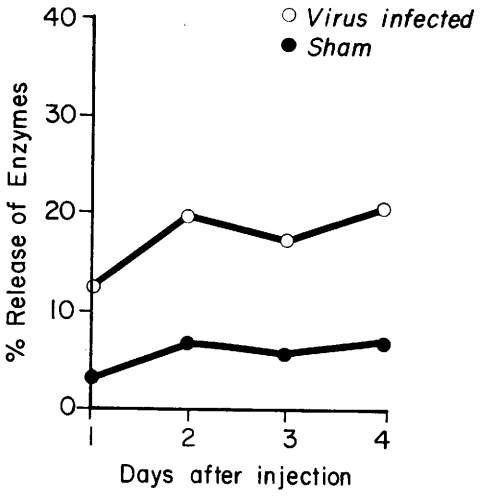

FIG. 3. Activity of free acid proteinase in liver homogenates of virus-infected and sham-control animals expressed as Free/(Free + Bound) × 100.

leaches out of the lysosomal membranes and is eventually discharged to the extracellular environment. Acid proteinase on the other hand showed a marked increase in free activity starting from the first day of the virus injection. This increase was not seen in the control animals. Allison and Sandelin (9) also found varying degrees of effect of murine hepatitis virus (MHV) on mouse liver lysosomal enzymes. They showed that acid proteinase was activated to a greater degree than acid phosphatase.

Increased lysosomal activation has been associated with cytopathic effects of virus infection by a number of authors including Ruebner and Hirano (10) and Wolf and Bubel (11). Walker and Pumper (12), however, did not find any strict correlation between cytotoxicity and lability of acid phosphatase in virus infected cells cultivated in serum and serum-free media infected with various viruses. Bartsch, Habermahl and Diefenthal (13) by protecting the lysosomes of virus-infected cells by guanidine showed that cytopathic effects are independent of the lysosomal release or activity.

Lysosomes are a heterogeneous group of subcellular organelles and the content of their enzymes varies not only between different tissues, but also in different cells of the same tissue (14). Acid phosphatase has been employed as a marker of lysosomal enzymes by biochemists and histologists mainly because of the ease with which it can be assayed or localized. The exact role that it plays in the metabolism of the cell besides attacking phosphate–ester bonds is still elusive. On the other hand, proteinase has a major role in the breakdown of the intracellular proteins, therefore, it would seem to find a meaningful

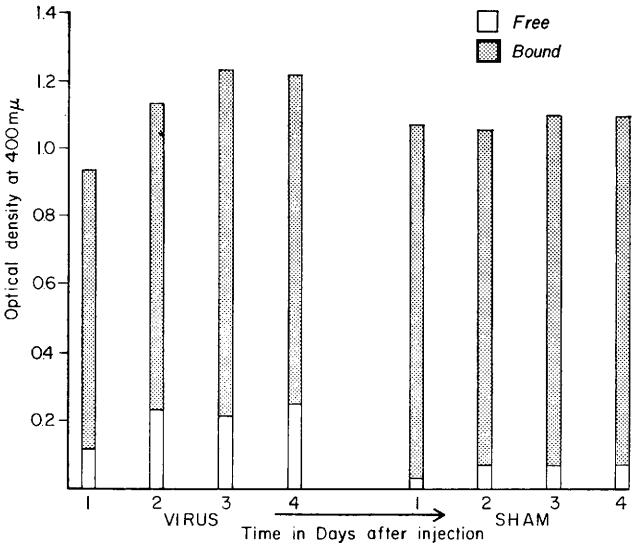

FIG. 4. O.D. of the reaction product of free and bound acid proteinase from virus-infected and sham-control animals.

correlation between proteinase activity than phosphatase activity in regards to viral cytopathogenicity.

Cholesterol and phospholipids constitute together integral and constant component of tissues the amount of which essentially remains unaltered by extreme changes in the nutritional status of the animal. The ratio of cholesterol-lipid phosphorus is constant for and characteristic of the tissue (15). Significance of the balance between phospholipids and cholesterol has been asserted as early as 1932 by Kooyman (16). The final degree of activity of a cell is determined by phospholipid–cholesterol ratio, and one-sided increase in the content of cholesterol of cells can arrest the physiological activity. It was observed by Vandenheuvel (17) that cholesterol forms associations with phospholipid. It is conceivable that cell-membrane structure is changed by increased amounts of cholesterol (18). Increased tissue cholesterol is also believed to cause essential fatty-acid deficiency (19). Dales (20) reported an increase in the level of phospholipids in Mengovirus-infected L-cells. In our study we observed that during MCMV infection the hepatic phospholipids stay constant, while the level of cholesterol increases by 100%.

This early increase in cholesterol may be due to congestion of bile canaculi, distended spaces of Disse and dense amorphous material (2).

Summary. Evidence is provided that during MCMV-infection in mice there is a different effect on different liver lysosomal enzymes. Acid phosphatase showed a variable degree of activation and seemed to play an insignificant role in the tissue breakdown. Acid proteinase on the other hand exhibited increased activation and appears to be partly responsible for MVMC-hepatitis. Total lipids and cholesterol increased several fold, while phospholipids remained constant.

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