LYMPHOID NECROSIS IN THE MOUSE SPLEEN PRODUCED BY MOUSE HEPATITIS VIRUS (MHV₃): AN ELECTRON-MICROSCOPIC STUDY

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EXPERIMENTAL infection of mice with murine hepatitis virus (MHV₃) has been shown to cause extensive necrosis of the lymphoid tissues of both spleen and lymph-nodes, in addition to widespread hepatic necrosis (Hirano and Ruebner, 1965).

Certain other viral infections have been associated with similar lymphoid destruction, such as infectious hepatitis experimentally induced in patas monkeys (Bearcroft, 1968), Gumboro disease in chickens (Cheville, 1967), distemper (Gibson, Griesemer and Koestner, 1965), feline panleukopenia (Hammon and Enders, 1939), yellow fever (Klotz and Belt, 1930), swine fever (Maurer, Griesemer and Jones, 1958), ectromelia (Mims, 1964), hog cholera (Seifried and Cain, 1932) and porcine rinderpest (Walter, 1964).

The mechanisms responsible for viral lymphoid necrosis remain undetermined; it is uncertain whether it is due to a direct viral assault on the lymphocytes, or is mediated through some viral-induced humoral mechanism, possibly of an immune nature.

The current ultrastructural study has attempted to contribute to the solution of this problem by looking for sites of virus proliferation within the spleen.

MATERIALS AND METHODS

Animals

Weanling (3–4-wk-old) pathogen-free female CD₁ mice (Charles River Laboratories, North Wilmington, Mass.) were used. They were caged in groups of ten and given food and water ad libitum.

Virus

The MHV₃ strain of murine hepatitis virus was used in the form of a 1 in 30,000 dilution in Gey's solution of liver suspension from infected animals.

 Procedures

Virus suspension (c. 100 LD₅₀ in 0.1 ml) was injected intraperitoneally into each of 60 mice. Thirty mice (group A) received a simultaneous intramuscular injection of 2.5 mg cortisol acetate (Merck, Sharp and Dohme, Philadelphia, Pennsylvania), and a similar daily dose of cortisol until death. The remaining 30 mice (group B) received only the virus.

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Ten mice (group C) were given neither virus nor cortisone, and provided a source of normal control material.

At 24 and 48 hr after the initial injection, three animals each from groups A, B and C were killed by cervical dislocation. At 72 hr a further three animals were killed in groups B and C, but only two in group A, in which they were the only survivors.

**Histological methods**

**Light microscopy.** Blocks of liver and spleen were fixed in Carnoy's fluid, embedded in paraffin, sectioned and stained with haematoxylin and eosin.

**Electron microscopy.** Small blocks of less than 1 mm³ were fixed in Millonig's osmic acid (Millonig, 1962), dehydrated in graded alcohols, cleared in toluene, and embedded in Araldite. Orientation of tissue was accomplished by initially viewing sections 1 μm thick stained with toluidine blue. Selected blocks were then cut on a Porter-Blum microtome to provide sections 15–25 nm thick. These were stained with lead citrate (Venable and Coggeshall, 1965) or uranyl acetate, mounted on copper grids and examined in an RCA EMU3G or 3H electron microscope. A total of 970 electron micrographs were taken for the study of 65 grids.

**RESULTS**

**Light microscopy**

The splenic changes observed in virus-infected (group-A) animals were similar to those reported by Hirano and Ruebner (1965). Structural alterations were not usually seen until 48 hr after virus injection, when the lymphocytes of each follicle underwent widespread pyknosis and karyorrhexis (fig. 1). In a few mice early changes were already present by 12 hr, and in these necrosis seemed to occur initially just internal to the marginal sinus. By 48–72 hr, macrophages within the marginal sinus and throughout the inner follicular zone were swollen and prominent, probably due to ingestion of cell debris. A fibrinous meshwork often developed in the peri-arteriolar area. Throughout this period the lymphoid elements of the marginal mantle were relatively well-preserved, forming a distinct ring around the "ghost" follicle. In the later stages plasma cells began to accumulate in this mantle zone.

In those mice given cortisone and virus (group B), Hirano and Ruebner's (1966) observation of relative protection of the lymphocytes was confirmed (fig. 2). The control mice showed no lesions.

**Electron microscopy**

The ultrastructure of the splenic white pulp in our control animals conformed closely to the description of Galindo and Imaeda (1962), with occasional branching macrophages and fixed reticular cells scattered amongst the large, medium-sized and small lymphocytes. The scanty cytoplasm of the lymphocytes contained numerous free ribonucleoprotein (RNP) granules but few organelles, mainly groups of mitochondria. Other cytoplasmic components included Golgi vesicles, multivesicular bodies, lipid inclusions and rounded dense bodies bounded by a single membrane, probably lysosomes (fig. 3). In the small lymphocytes only a very few profiles of smooth endoplasmic reticulum (ER) were present, whilst in the larger lymphocytes a few small sacs of rough
Fig. 1.—Lymphoid follicle in spleen of CD1 mouse given an intraperitoneal injection of MHV3 virus 48 hr previously. Note extensive pyknosis and karyorrhexis of lymphocytes surrounding central arteriole. 1μm section. Toluidine blue (TB). × 375.

Fig. 2.—Lymphoid follicle in spleen of virus-infected CD1 mouse also given daily injections of cortisone acetate. Note striking preservation of lymphocytes as compared with fig. 1. A central arteriole is not represented in this plane of section. 1μm section. TB. × 375.

Fig. 3.—Electron micrograph (EM) of lymphocytes of splenic white pulp in normal control CD1 mouse showing high nuclear : cytoplasmic ratio. Free ribosomes and mitochondria are the predominant features in the scanty cytoplasm, but note also several dense lysosome-like bodies (arrows) in two cells. Only a few small profiles of endoplasmic reticulum can be seen. A nuclear body (double arrow) is recognisable in one nucleus. × 17,600.
**FIG. 4.**—EM of the splenic follicle illustrated in fig. 2. A lymphocyte shows numerous virus particles (arrows) within the cisternae of the ER. Note the reticular inclusion (double arrows). ×18,500.

**FIG. 5.**—Adjacent area to that in fig. 4. One cell shows complex whorl formations (→) of ER and an increase in free ribosomes. There is also an enlarged mitochondrion with many irregularly arranged cristae (M). No virus particles are seen in this cell, which probably shows a late stage of infection. EM. ×11,000.

**FIG. 6.**—Higher magnification of virus particles in a lymphocyte within dilated sacs of rough ER. Budding is occurring from the ER (→) and a clustering of budding forms around a core of ribosomes is visible (C). EM. ×45,000.

**FIG. 7.**—Splenic red pulp. A few virus particles (arrows) are shown within the endoplasmic reticulum of a reticular cell lining a sinusoid (S). Note the basement membrane (BM). EM. ×13,900.
ER were also seen. Nuclear bodies were observed with great frequency within the nuclei of both lymphocytes and reticular cells. Rarely, a lymphocyte was found in mitosis or undergoing necrosis. The macrophages usually contained small quantities of phagocytosed material in their cytoplasm. Around each follicle ran a marginal sinus receiving blood from capillaries that originated from the central arteriole. Peripheral to the marginal sinus was the marginal zone consisting mainly of medium-sized and large lymphocytes. No viral particles were found in the controls.

In the virus-infected mice of group A there was usually no change in the normal splenic structure pattern during the first 24 hr after infection. By 48 hr, however, the majority of the lymphocytes in the follicles had become necrotic. Viral particles could not be demonstrated within the necrotic lymphocytes, perhaps because of the very rapid progression of the necrotic process. At one stage the lymphocytes all appeared morphologically normal, whilst at the next most were represented by shrunken, densely osmiophilic and often fragmented material lying in a matrix of fibrin strands, free organelles and macrophages. Even the few surviving lymphocytes in the necrotic areas usually maintained a normal architecture. On the other hand, in the infected mice which were also treated with cortisone (group B) intracellular viral particles were identified within a small number of lymphocytes (approximately 2–5 per cent.) in most of the lymphoid follicles (fig. 4). The great majority of the lymphocytes were severely necrotic and without detectable viral particles. It was our impression that a considerable proliferation of rough ER preceded the appearance of recognisable virus in infected lymphocytes. A few lymphocytes in virus-forming areas showed a considerable increase in the amount of both rough and smooth ER, with a tendency to complex whorl formations as well as abnormal swollen mitochondria with numerous tortuous criss-crossing cristae but without identifiable viral particles (fig. 5). It is possible that these cells represented a late stage of infection. In the areas of virus formation early crescentic viral particles could be seen acquiring their outer coats by budding from the rough ER (fig. 6), and occasionally several budding forms clustered around a core of ribosomes in a “flower petal” arrangement (Ruebner, Hirano and Slusser, 1967). Most viral particles were found within dilated sacs of the proliferated rough ER. The zone between outer and inner nuclear membranes became irregularly widened and this space sometimes contained a few viral particles. In one lymphocyte a small reticular inclusion was found adjacent to the viral particles (fig. 4). Despite the presence of viral particles the morphology of the lymphocytes, at this stage of infection, was not greatly altered, apart from some increase in the density of the mitochondria. The severely damaged cells without viral particles were sometimes characterised by margination and increased density of the nuclear chromatin, with mitochondrial swelling, but more frequently by shrinkage and intense osmiophilia of both cytoplasmic and nuclear components.

Extracellular viral particles were seen commonly in animals of both groups A and B. Lead-stained sections demonstrated the morphology of the viral particles more clearly than sections stained with uranyl acetate.
The red pulp was not studied exhaustively. Scattered necrotic cells were found within cords and sinuses. A few reticular cells contained virus particles within their ER (fig. 7). However, neither necrosis nor viral budding was observed in reticular cells and macrophages. These cells seemed more resistant than lymphocytes, and there were no obvious alterations of their nuclear chromatin.

DISCUSSION

Involvement of the lymphoid tissues is a common histological finding in many virus infections. In some diseases, such as measles and rubella, this takes the form of a lymphocytic and reticulum-cell hyperplasia, but in others, like mouse hepatitis (Hirano and Ruebner, 1965) and ectromelia (Mims, 1964), necrosis of lymphocytes in the major lymphoid organs is a prominent feature. It therefore seems probable that lymphoid tissue plays a part in the progression of these infections. However, the underlying cellular mechanisms remain obscure in spite of much speculation (Mims).

The current study of MHV3 infection as a model demonstrated virus formation (budding) within small lymphocytes of the splenic white pulp, strongly suggesting that the lymphoid necrosis, at least in MHV3 infection, is related to the presence of intracellular virus. The relative scarcity of virus particles within these cells was probably due to the rapidity with which lymphocytes degenerate after infection. It is noteworthy that intracellular viral particles were found only in the spleens of the cortisone-treated mice. This may be due to the stabilising effect of the cortisone on lysosomal and cellular membranes (Weissmann and Thomas, 1964), which might also account for the less rapid destruction of the follicles in these animals (Hirano and Ruebner, 1966). Mims, studying ectromelia infection, believes that splenic macrophages are an important site of viral replication, but although we found MHV3 virus particles within some macrophages and reticular cells, budding could not be demonstrated. Clarification of any time-relations in the infection of the various cell-types in the spleen was not possible in this study.

As in the liver (Hirano and Ruebner, 1965), a necessary prerequisite for MHV3 replication seems to be an accompanying proliferation of rough ER, which is then utilised for virus formation by budding. This proliferation of rough ER suggests an increased protein synthesis for virus production by the lymphocytes. Whether it is also associated with antibody production is unresolved at present. Although later transitional forms of lymphocyte-plasma cell transformation were not observed during the current experiment, plasma cells have been recognised in the marginal zones and occasionally amongst the cellular debris of follicles destroyed during the late stages of MHV3 infection, and are thought to represent transformed lymphocytes (Hirano and Ruebner, 1965).

The reticular inclusion found in the cytoplasm of an infected small lymphocyte may be an essential stage in the synthesis of viral nucleoprotein, as suggested by David-Ferreira and Manaker (1965) who found identical inclusions in tissue-cultured hepatocytes infected with MHV3. However, examination of mouse
lymphoid necrosis in mouse hepatitis virus failed to reveal similar structures, though dense ribosomal aggregates near virus formation areas were observed (Ruebner, Hirano and Slusser, 1967).

Although the MHV3 group of viruses does not appear to be related to human viral hepatitis it is of interest that splenic involvement also occurs in that disease. Palpable splenomegaly has been observed in 16.5 per cent. of one series of 200 cases, although there was no obvious correlation with the severity of hepatic damage (Wilson, 1951). This suggests that the splenic enlargement may have been a separate manifestation of the infection rather than secondary to portal venous congestion. In another series of fatal human cases of infectious hepatitis histological study showed necrosis of the germinal centres of the splenic white pulp to be an almost constant feature (Bearcroft, 1968). Similar degeneration of cells in both the red and white pulp has been recorded in patas monkeys into which material from cases of infectious hepatitis had been injected (Bearcroft).

**Summary**

Pathogen-free CD1 mice were experimentally infected with mouse hepatitis virus (MHV3), and half of them were also given daily doses of cortisone. Ultrastructural examination of the splenic white pulp revealed viral particles, corresponding in size, shape and structure to MHV3, within small lymphocytes as well as extracellularly. Virus formation within the lymphocytes was associated with proliferation of the rough endoplasmic reticulum and budding of viral particles from these membranes. Intracellular viral particles were readily seen only in the cortisone-treated mice, possibly because of the stabilising effect of cortisone on intracellular membranes. It is suggested that the lymphoid necrosis of MHV3 infection is attributable to the virus replication within the lymphocytes.

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