

# Herpes simplex virus hepatitis in macrophage-depleted mice: the role of massive, apoptotic cell death in pathogenesis

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Infection with herpes simplex virus or hepatitis viruses can lead to fulminant hepatitis, but there is controversy about the underlying conditions needed for such disease. To investigate how the impairment of host defences might be involved, macrophages were depleted by administration of silica to mice before intravenous injection with herpes simplex virus type 1 (HSV-1). Such mice died rapidly and their livers were yellowish and shrunken (acute yellow atrophy), and occasionally grossly haemorrhagic. Small foci of apoptotic cells developed in the liver lobules; these rapidly became

confluent and zonal over time. The overall lesion pattern was similar to massive hepatic necrosis, and there was extensive HSV replication in the liver lesions. In the liver, DNA fragmentation characteristic of apoptosis followed the time course of HSV-1 propagation. These findings suggest that one of the underlying conditions for fulminant viral hepatitis may be inadequate macrophage response, and that the massive hepatic damage, often defined as cell necrosis, may actually be apoptosis of liver cells subsequent to virus infection.

## Introduction

Fulminant hepatitis, a fatal disease syndrome of severe liver parenchymal damage, may have various aetiologies such as hepatitis viruses, drugs, halothane, and shock (circulatory collapse) (Berk & Popper, 1978). It is well known that in particular circumstances herpes simplex virus (HSV) can also cause fulminant hepatitis. The morphological changes in massive hepatic cell death are similar regardless of the causal agent (Cockayne, 1912; Lucke, 1944; Popper & Franklin, 1948; O'Brien & Gottlieb, 1982) except in specialized cases (Kiotz & Belt, 1930; Zimmermann, 1981). The liver becomes yellowish and shrunken, or in some cases haemorrhagic. Massive hepatic cell death develops between 24 h and 4 weeks following the onset of clinical illness (O'Brien & Gottlieb, 1982). There is still much controversy about the underlying conditions related to

fulminant hepatic pathological changes. For example, herpetic hepatitis is frequently associated with burns, cancer, pregnancy or renal transplantation conditions in which host defences are known to be diminished (Flewett *et al.*, 1969; Foley *et al.*, 1970; Borhanmneesh *et al.*, 1973; Goyette *et al.*, 1974; Anuras & Summers, 1976; Chase *et al.*, 1987). Such underlying conditions are considered to include an impairment of host defences, especially lymphocyte dysfunction.

Herpetic hepatitis in mice can be exacerbated experimentally by intravenous administration of silica before infection with virus (Allison *et al.*, 1966; duBuy, 1975; Mogensen & Andersen, 1977; Schirmacher *et al.*, 1989). In this model system, macrophage blockade plays a major role in the exacerbation of hepatic cell death. However, in humans it is not clear whether lymphocyte suppression or functional deficiency of macrophages may play a critical role.

In fulminant hepatitis, hepatocytes die in massive numbers – at present the mechanisms leading to such cell death are not clear. How hepatocytes die in such a short time, even within

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24 h, may be due to acute phenomena such as the Schwartzman reaction, circulatory shock or other circulatory disorders, or immunological disturbances such as the Arthus reaction (Sabensin, 1963; Mori *et al.*, 1981; Irie & Mori, 1986). Recently, Fas and Fas ligand were reported to be involved in this massive liver cell death and a link has been suggested between apoptosis and fulminant hepatitis (Ogasawara *et al.*, 1993; Galle *et al.*, 1994; Ando *et al.*, 1993; Nagata & Golstein, 1995). The present study indicates that in the mouse, in the absence of functional macrophages, infection with HSV in the liver can lead to massive apoptosis.

## Methods

■ **Experimental animals and virus inoculation.** C3H/HeN male mice were obtained from Charles River Laboratories and used at 8–10 weeks of age. HSV-1 (Miyama + GC strain) (Nii & Kamahora, 1961) was supplied by Kumagai, Tohoku University School of Dentistry, Sendai, Japan. The virus was grown in green monkey kidney (GMK) cells and stored at  $-80^{\circ}\text{C}$ . Virus was diluted in PBS before use to give  $1 \times 10^6$  p.f.u. in 0.2 ml which was injected into mice via the tail vein.

■ **Reagents.** Anti-thymocyte serum and anti-asialo GM1 serum were purchased from Wako Pure Chemicals. Silica (particle size  $0.014 \mu\text{m}$ ) was obtained from Sigma. The powder was suspended at 10 mg/ml in PBS, sterilized at  $100^{\circ}\text{C}$  for 20 min and stored at  $-20^{\circ}\text{C}$  until used.

■ **Silica treatment.** Treatment was as described previously (Harada *et al.*, 1989; Irie *et al.*, 1992). Briefly, mice were injected intraperitoneally (i.p.) with silica, 2 mg/0.2 ml, 1 day before intravenous (i.v.) injection of HSV. The efficacy of the macrophage blockage was assessed as described by Hirsch *et al.* (1969) with some modifications. In brief, 24 h after silica treatment, mice were injected intravenously with 2 mg each of a colloidal carbon suspension (at 10 mg/ml). Blood samples were collected at 5 and 15 min after injection and the red cells lysed in 0.1%  $\text{Na}_2\text{CO}_3$ . The samples were read in a spectrophotometer at 660 nm and the readings converted to mg carbon/ml blood in comparison with known standards. Phagocytic indices (*K*) were determined by standard methods. The average *K* value of three mice treated with silica was  $0.013 \pm 0.0012$  compared with an index of  $0.0254 \pm 0.009$  in three untreated animals, a significant difference ( $t = 9.78$ ).

■ **Depletion of natural killer cells and T lymphocytes.** To deplete NK cells, mice were injected with 0.2 ml anti-asialo GM1 serum once daily for 3 days before injection of virus. The NK activity of spleen cells was completely suppressed as shown by the target assay with  $^{51}\text{Cr}$ -labelled YAC cells (data not shown).

To deplete T lymphocytes, 0.2 ml antithymocyte serum was given i.p. daily for 3 days before virus injection. In such animals, the lymphocyte proliferation response to the mitogens con-A and PHA was abolished, whereas responses to the B cell mitogen LPS were unaffected (data not shown).

■ **Liver enzyme assays.** Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured according to the method of Bergmeyer (1984).

■ **Light microscopy and electron microscopy (EM).** For light microscopy, the livers were fixed in 10% buffered formaldehyde, embedded in paraffin wax and sections stained with haematoxylin and eosin. For EM, the liver was fixed in 2.5% glutaraldehyde, post-fixed with osmium tetroxide and stained with uranyl acetate and lead citrate.

Sections were observed by transmission electron microscopy (JEM, 1200 EX).

■ **Assay of infectious virus in tissues.** The method of assay has been described previously (Irie *et al.*, 1992). Briefly, the liver was removed, aseptically homogenized with a glass homogenizer and sonicated (B-32 sonicator, Branson) for 5 min. After centrifugation at 2500 r.p.m. for 5 min, the supernatants were diluted to a 10% (w/v) mixture with RPMI 1640 medium supplemented with 2% foetal bovine serum and kanamycin (100 ml/ml). Confluent monolayers of GMK cells in 24-well plates were inoculated with  $\log_{10}$  dilutions. After adsorption of the inoculum for 1 h at  $35^{\circ}\text{C}$ , the wells were replenished with medium containing methylcellulose and incubated at  $35^{\circ}\text{C}$  for 2 days. Plaques were counted after fixing the cells and staining with crystal violet.

■ **Detection of DNA fragmentation.** DNA was extracted from livers by the method of Hirt (1967) with some modifications. Briefly, about one-tenth of the liver tissue from a mouse was minced and lysed by adding 400  $\mu\text{l}$  lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM EDTA) containing 0.6% sodium lauryl sulphate. Cell lysates were gently mixed with 125  $\mu\text{l}$  of 5 M NaCl and kept at  $4^{\circ}\text{C}$  overnight. The mixture was centrifuged at 14000 *g* for 30 min and the chromatin pellet was then removed. After treatment with RNase and proteinase K, the DNA in the supernatant was precipitated with ethanol and resuspended in TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA). Aliquots were then analysed for nucleosomal DNA fragments by electrophoresis on a 1.5% agarose gel.

An ELISA method kit (Leist *et al.*, 1994) was used to assay the amount of DNA fragmentation. For this a whole liver was suspended in 5 ml PBS and disintegrated by three strokes with a glass homogenizer. About 20% of this homogenate was centrifuged at 13000 *g* for 15 min and the supernatant used for the assay. The assay was based on the quantitative sandwich enzyme immunoassay (ELISA) principle using two mouse monoclonal antibodies directed against DNA and BrdU (5-bromo-2'-deoxyuridine), respectively. This allowed the specific detection and quantification of BrdU-labelled DNA fragments. Data were expressed as original optical density (OD) reading in milli OD (mOD) (means  $\pm$  SEM).

## Results

Before intravenous injection with HSV-1, groups of mice were treated to deplete either macrophages (with silica treatment), NK cells (with antibody to asialo GM1) or T cells (with antibody to thymocytes) and a control group was given PBS alone. Animals were observed daily for clinical signs of disease and deaths. At different times after infection, blood samples were taken for analysis of ALT and AST levels and groups were killed for examination of the liver by histology (light microscopy and EM), assay of infectious virus and DNA fragmentation.

### Clinical, pathological and histological observations

The mean survival periods, in days, of the PBS-, anti-thymocyte serum-, anti-asialo GM1- and silica-treated mice were  $4.7 \pm 1.05$ ,  $5.1 \pm 1.6$ ,  $3.1 \pm 0.64$ , and  $1.6 \pm 0.78$ , respectively. The survival period of the silica-treated mice was significantly shorter than that of the other three groups ( $t = 3.01$ ,  $P < 0.01$ ). After challenge with virus, the PBS-treated

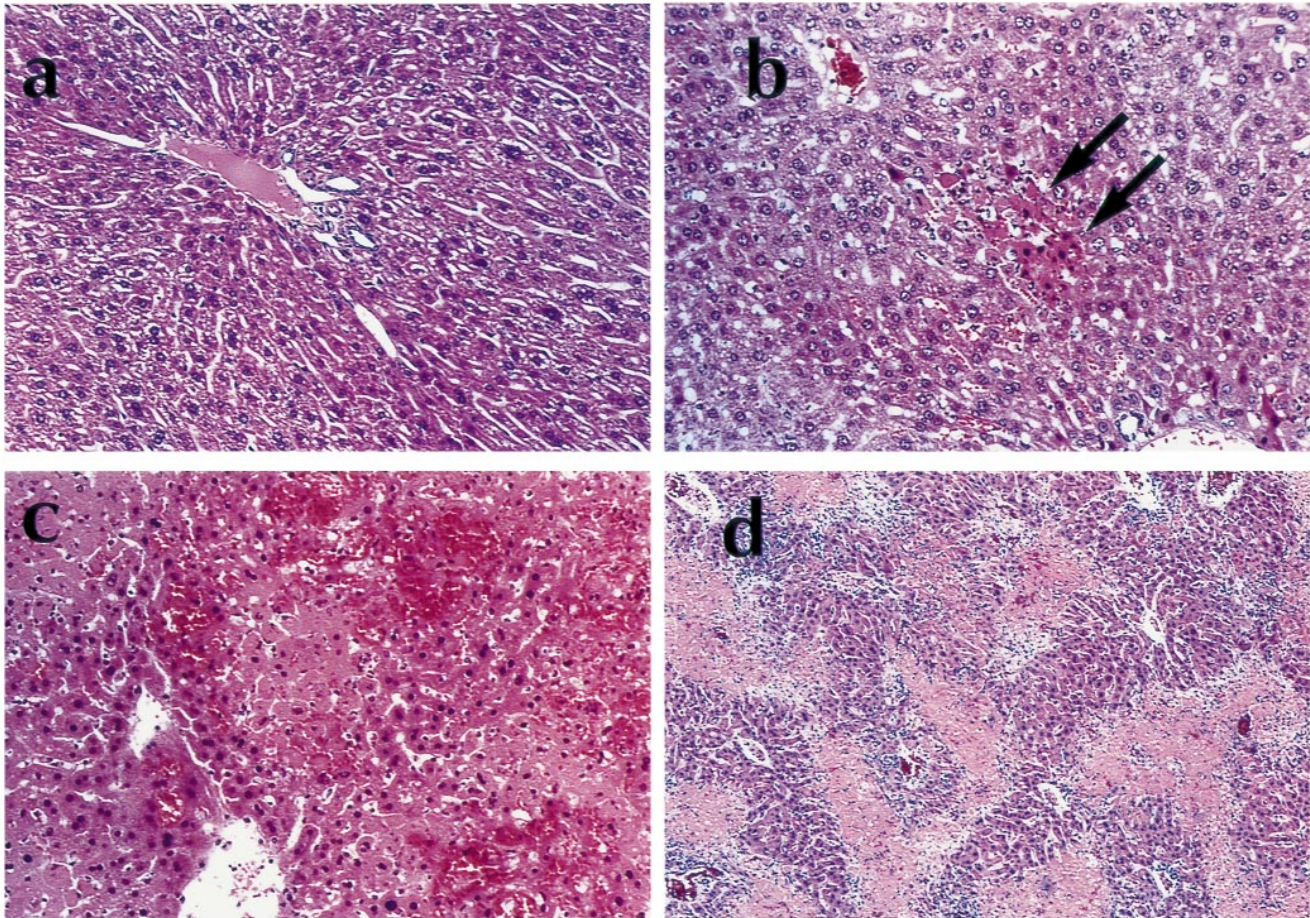


Fig. 1. Histology of the liver; all sections stained with haematoxylin and eosin. (a) From an uninfected mouse ( $\times 100$ ). All others from silica-treated animals after i.v. inoculation of virus: (b) 3 h after inoculation of virus, showing small foci of dying cells with fragmented nuclei in the intermediate zone of the liver lobule (arrows) ( $\times 100$ ); (c) 12 h after virus inoculation, foci of dying cells increased in number, with numerous apoptotic cells at the border between remaining hepatocytes and central area of lytic necrosis ( $\times 100$ ); (d) 24 h after virus inoculation the foci have become confluent to form areas of massive zonal hepatocyte death ( $\times 40$ ).

mice and those given anti-thymocyte serum were normal for 4 days and some became immobile after 5 days, in some cases developing convulsions. In contrast those given silica or anti-asialo GM1 became immobile after 24 h and all mice had died by 3 or 4 days. Uninfected mice given the above treatments all remained normal.

After silica treatment, the livers of HSV-infected mice were yellowish and shrunken or, in some cases, swollen and reddish with gross haemorrhage. Sequential histological examination revealed small foci of apoptotic cells in the intermediate zone of the liver lobule within 3 h of infection (Fig. 1*b*, arrows), increasing further over the next 6 h. Lesions at the centre of the lobule became confluent and zonal 12 h after infection (Fig. 1*c*). Furthermore, lytic necrosis was observed at the centre of the lesion. EM confirmed the presence of cells with characteristic nuclear signs of apoptosis (severe clumping of chromatin and dissociated fragmentation of nuclei, Fig. 2). At high magnification it was clear that infected nuclei were packed with many

virus capsids lacking dense cores (Fig. 2, inset). Few apoptotic cells were seen by EM in the parenchyma of mice not treated with silica from 1 to 3 days after infection.

At 24 h, five of six animals examined showed liver changes similar to so-called massive hepatic necrosis (defined here as grossly yellow or haemorrhagic livers with confluent zonal or panlobular necrosis over half the histological section) (Fig. 1*d*, Table 1). However, no inflammatory cells were detectable in the vicinity of these lesions. Few apoptotic cells were found in parenchyma of the livers of control, anti-thymocyte serum or anti-asialo GM1-injected mice.

Except for one mouse on day 3 after infection in the group treated with anti-asialo GM1, these severe hepatic lesions were not observed in the control or other groups or in uninfected animals given the depletion treatments alone. Uninfected mice treated with silica remained clinically normal and did not show histological abnormalities in the liver when examined by light microscopy and EM.

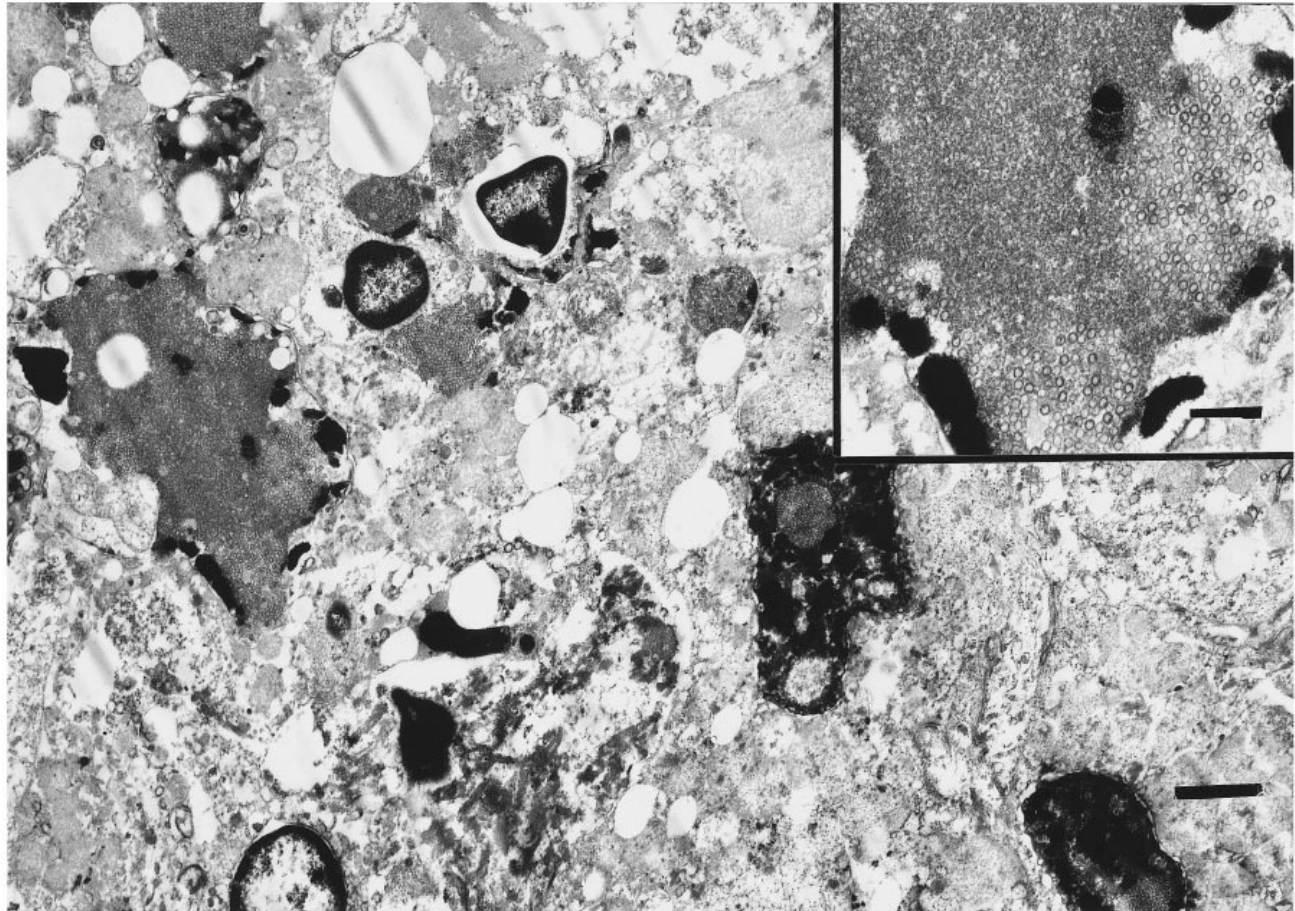


Fig. 2. Electron micrograph of a liver lesion from a silica-treated mouse 12 h after i.v. inoculation with HSV. Cells contain condensed and fragmented nuclei characteristic of apoptosis (bar, 2  $\mu$ m). Insert, higher magnification of same preparation showing numerous viral capsids without dense cores (bar, 500 nm; uranyl-lead nitrate stain).

### Serum ALT and AST levels

In mice treated with silica prior to infection, serum ALT and AST levels were  $576 \pm 113$  and  $344 \pm 94$  units/l, respectively, at 3 h after infection. Corresponding values in non-treated infected mice were  $103 \pm 81$  and  $54 \pm 7$  units/l, respectively. At 24 h, levels of these enzymes were  $5086 \pm 542$  and  $3130 \pm 483$  units/l, indicating marked impairment of liver function. Uninfected mice treated with silica did not show the abnormal levels of these liver-specific enzymes in the serum.

### Virus replication in the liver

In control mice, virus titres reached a peak at about 12 h but fell to undetectable levels by 24 h (Fig. 3*a*). Similar results were obtained in mice treated with anti-thymocyte serum or anti-asialo GM1 (data not shown). In contrast, mice given silica had virus titres in the liver 2 logs higher than controls at 12 h, and these high levels persisted over the next 2 days.

Infectious virus was not detected in the brain and brain stem of mice treated with silica until 3 days after infection (data not shown).

### DNA fragmentation

Gel electrophoresis showed DNA fragmentation in livers of silica-treated mice as early as 3 h after infection with virus, but this fragmentation was more marked at 24 h (Fig. 4). No such effects were detected in uninfected livers of silica-treated and untreated control mice. As shown in Fig 3, the time course of quantitative DNA fragmentation was similar to the kinetics of HSV-1 propagation in the silica-injected mice.

Some fragmentation was observed at early times after infection in the absence of silica treatment but it declined thereafter and was undetectable at 24 h.

### Discussion

Following intravenous inoculation, viruses such as HSV are rapidly taken out of circulation (Hill *et al.*, 1986), presumably by cells of the reticuloendothelial system in organs such as the liver. The present study confirms other work (Allison *et al.*, 1966; duBuy, 1975; Mogensen & Andersen, 1977; Zisman *et al.*, 1970) which shows that if this system is compromised, in particular if macrophages are depleted by silica treatment

**Table 1.** Incidence of massive hepatic necrosis after intravenous inoculation of HSV in mice depleted of macrophages, T lymphocytes or NK cells or untreated control mice

Massive hepatic necrosis is defined as grossly yellow or haemorrhagic livers with confluent zonal or panlobular necrosis over half the histological section. Values shown are the number of positive animals/total number of mice examined.

Treatment	Time after infection (days)			
	1	2	3	4
None*	0/6	0/6	0/6	0/5
Anti-thymocyte serum†	0/6	0/6	0/6	0/5
Anti-asialo GM1‡	0/6	0/6	1/6	0/5
Silica§	5/6	6/6	4/4	ND

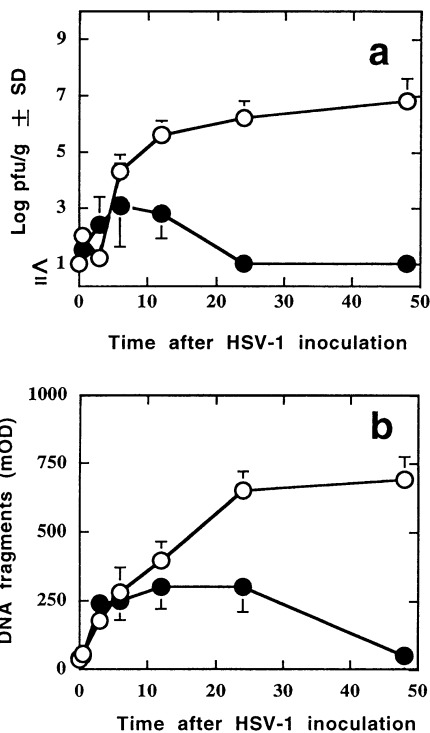
ND, Not done.

\* Not treated (infected with HSV-1 only).

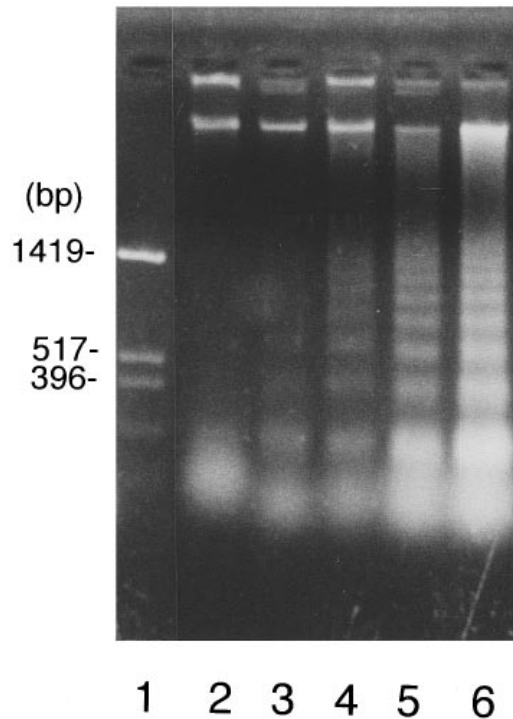
† 0.2 ml × 3 intraperitoneal administration before HSV-1 intravenous inoculation ( $1 \times 10^6$  p.f.u.).

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§  $\phi$  0.014  $\mu$ m. 2 mg/0.2 ml × 1 intraperitoneal administration 1 day before HSV-1 intravenous inoculation ( $1 \times 10^6$  p.f.u.).



**Fig. 3.** (a) HSV-1 replication in the liver of silica-treated mice (○) and untreated mice (●) after i.v. inoculation of HSV. (b) DNA fragmentation in livers of such mice. Each point (and its standard deviation) was derived from results from three to four mice.



**Fig. 4.** DNA fragmentation in liver of mice treated with silica before i.v. inoculation of HSV. From an uninfected mouse (lane 2); 3 h after inoculation (lane 3); and after 6 h (lane 4); 12 h (lane 5); 24 h (lane 6). Lane 1 contains size markers.

before infection, the virus can establish extensive infection in the liver tissue. This, and the lack of a similar effect after depletion of T lymphocytes, confirms the importance of the macrophage as an early defence barrier against this virus. Moreover, the enhancement of liver infection following silica treatment is not restricted to HSV since similar observations have been made with yellow fever (Zisman *et al.*, 1971) and rabies viruses (Turner & Ballard, 1976) and with vesicular stomatitis and influenza A viruses (our unpublished observations).

A striking feature of the present study was the high incidence, rapidity and extent of the massive hepatic cell death which occurred after macrophage depletion. Moreover, this severe liver damage was paralleled closely by extensive virus replication in the liver, raised levels of liver enzymes in the serum and fragmentation of DNA (characteristic of apoptosis) in the infected liver. The single case of such severe damage in mice depleted of NK cells may have arisen because of a partial macrophage blockade which can follow such depletion (Schirmacher *et al.*, 1989).

The rapid onset of massive hepatic cell death in macrophage-depleted mice, and the lack of virus in the brain of such animals until day 3 after infection, suggests that their short survival time (a mean of about 1 day) was a direct result of the liver damage rather than encephalitis. Moreover, these animals lacked the clinical signs indicative of encephalitis, such as

convulsions, that were evident in the controls or those treated with anti-thymocyte serum. Similar effects of silica on mortality and survival time after HSV infection were found by Zisman *et al.* (1970) but not by Mogensen & Andersen (1977).

The earliest histological signs of virus infection in the liver of macrophage-depleted mice were in the intermediate zone. This may simply reflect the easier access of virus to liver cells at this site, since here the sinusoids have no basement membrane.

A further striking feature of the severe liver damage after silica treatment, revealed by DNA analysis and microscopy, was the presence of extensive apoptosis in the infected areas of the tissue. This apoptosis was clearly associated with the virus infection since the extent of DNA fragmentation and the amount of virus replication followed very similar dynamics. Reports on the association of apoptosis and HSV infection indicate that its occurrence may depend on a number of factors such as cell type and viral gene expression. For example, in productive infection of 3T3 (Razvi & Welsh, 1995) and Hep2 (Koyama & Miwa, 1997) cells in culture, apoptosis was not detected. More recent observations in Vero cells in culture indicate that the virus can induce apoptosis but its occurrence, at least in these cells, is blocked by the immediate early protein ICP4 (Leopardi & Roizman, 1996). However, apoptosis has been reported in peripheral blood mononuclear cells infected with the virus (Tropea *et al.*, 1995; Ito *et al.*, 1997), and in infected neurons and glial cells of the spinal cord and infected glial cells in sensory ganglion of mice infected with HSV-2 (Ozaki *et al.*, 1997). Moreover, apoptosis has also been seen in uninfected stromal keratocytes underlying HSV-infected epithelial cells of the cornea (Wilson *et al.*, 1997). In this situation the response is probably an indirect effect of the infection, perhaps mediated by cytokines, and it has been suggested to be a defence mechanism to stop spread of the infection into deeper layers of the cornea.

As suggested for the cornea, apoptosis in infected livers may also represent a primitive defence against the virus, but in the liver it is the infected cells themselves that are mainly involved. The DNA degradation associated with apoptosis can involve both that of the host cell and the virus. In this respect, it is noteworthy that the nuclei of the infected hepatic cells were packed with many viral capsids which lacked the dense cores normally associated with the DNA component. Such particles would presumably be non-infectious and thereby the spread of infection from the liver could be diminished. In this respect it is noteworthy that when apoptosis was induced by chemical treatment of Hep2 cells, HSV infection in these cells was restricted (Koyama & Miwa, 1997).

Under normal circumstances, with macrophages operating as the first line of defence to control infection in the liver, the apoptotic response would be less obvious. However, in the absence of this defence, following silica treatment, the widespread infection and associated apoptotic response would explain the rapid fulminant hepatitis observed in the present

studies. It is therefore of interest that several recent reports indicate a role for apoptosis in fulminant hepatitis (Ogasawara *et al.*, 1993; Galle *et al.*, 1994; Ando *et al.*, 1993; Nagata & Golstein, 1995). The histological type of the massive hepatocyte death reported here was confluent, zonal and mainly centrilobular. This type of massive centrilobular hepatic death has long been considered to be due to circulatory disturbances of the liver such as shock, disseminated intravascular coagulation or the Schwartzman reaction. Anoxia may play a role in this pathogenesis, since oxygen pressure is reported to be high in the blood of periportal areas but low in centrilobular areas, possibly rendering the centrilobular areas more sensitive to anoxia. On the other hand, apoptosis appears to be part of the regenerative processes in the liver, especially in the centrilobular area after the migration of senescent hepatocytes from the periportal to the centrilobular area (Benedetti *et al.*, 1988). In addition, apoptotic cells are found even in the periportal area in hepatocytes recovering from ethanol-induced liver damage (Zajicek *et al.*, 1985). Considering these findings, it would appear that hepatocytes in the intermediate to centrilobular area are likely to be more sensitive to apoptotic death than those in the periportal area.

In summary, it is suggested that deficient macrophage function is one of the important underlying conditions for fulminant viral hepatitis, and the massive death of hepatic centrilobular cells observed in this study is similar to that observed in massive hepatic necrosis caused by circulatory disturbance in humans. To our knowledge, this is the first study to show that apoptosis plays a major role in the massive hepatocyte death induced by HSV-1 and provides a further example of a tissue in which apoptosis may be induced by this virus.

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