MECHANISMS OF HEPATIC INJURY IN MURINE HEPATITIS VIRUS TYPE 3 INFECTION

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ABSTRACT

Murine hepatitis virus type 3 (MHV-3), a member of the coronavirus family, induces a response that varies with the age and genetic background of the host mouse strain. A/J mice are fully resistant to the virus, while Balbc/J are fully susceptible and C3HebFe/J are semi-susceptible, making it possible to predictably reproduce the major human responses to hepatitis viruses. Although there has been considerable discussion of viral pathology in the literature, there has been much less emphasis on pathogenesis. In the experiments described here, histological, biophysical, and immunological techniques have been used to define the processes and cells involved.

Transmission electron microscopic observations have confirmed that Kupffer and endothelial cells of hepatic sinusoids show clear changes by 12 hrs post-infection (p.i.), which are more advanced than hepatocellular changes. No replicating virus was seen in altered hepatocytes up to 3 days p.i. Scanning electron microscopy demonstrated that areas of necrosis are focal in nature and at 2-3 days p.i. consist of small spherical areas without flow. In vivo microcirculatory studies confirm the localized nature of the lesion and have shown that red cell velocity can be recorded in individual sinusoids . Velocities were found to vary from zero within a lesion to a normal velocity of 69 ± 31 um/sec over a distance of not more than 3 sinusoids. In-vivo microcirculatory studies also revealed the ability of macrophages to move upstream (against flow) in the hepatic sinusoids.

Using fluorescein labelled antibodies to cell surface markers (Thy-1, Lyt-2, and L3T4) it was shown that no T-cells of any subset were present in the areas of hepatocellular necrosis. Furthermore, treatment with cyclosporine A, which would be expected to decrease necrosis due to cell mediated cytotoxicity, did not significantly alter the course

of the disease. The only cells which increased in number in the liver post infection were cells of the monocyte/macrophage lineage (Mac 1+), which had increased twofold at 12 hrs ($p<.025$) p.i. and to greater than twenty fold ($p<.005$) by 3 days p.i.

Resistance in the A/J strain did not reflect an inability of the immunocompetant cells to present and respond to viral antigen. It was demonstrated that MHV-3 infected macrophages from resistant A/J mice are better able to stimulate proliferation of allogeneic and syngeneic lymphocytes than those from the sensitive Balb/cJ strain. In contrast, MHV-3 infection caused a significant enhancement of chemiluminescence from Balb/cJ macrophages, which did not occur in A/J animals.

In vivo studies demonstrated a significant increase in free radical reaction products, including conjugated dienes (of long chain free fatty acids and aldehydes), thiobarbituric acid reactive substances, and lipid soluble fluorescent products between 12-72 hours p.i. with MHV-3 in the livers of susceptible Balb/cJ strain mice. All of these are products of oxidative cleavage of cellular and membrane polyunsaturated fatty acids, and result from the action of oxygen free radicals. Free radical inhibitors, or quenchers of free radical reaction products, were able to significantly reduce the liver necrosis in the susceptible mouse strain following infection.

Radioimmune assays for antibody to MHV-3 have confirmed the presence of preformed antibodies to (or cross-reactive with) MHV-3 in the sera of both susceptible and resistant mice, pre and post-infection. Immunofluorescent labelled antibodies have also been used to demonstrate the presence of IgG deposits in the sinusoids of the liver both pre and post infection. This suggests the possibility that these mice have been infected with a non-virulent MHV strain prior to these experiments.

From these studies, we conclude that the hepatic injury caused by MHV-3 infction in Balb/cJ mice is mediated predominantly by fixed and migratory cells of the mononuclear phagocytic series. Susceptibility and resistance are related to strain dependant differences in the response of macrophages (and Kupffer cells) to infection, and include the release of procoagulant activity (previously shown) and reactive oxygen radicals (and possibly other macrophage activation products such as PAF) that act together to induce hepatocellular necrosis. Preformed non-neutralizing antibody and an intact complement cascade may enhance viral uptake and activation of macrophages in the Balbc/J mice. Resistance to necrosis may be enhanced by a genetic deficiency of C5 in the A/J mice, preventing the formation of the membrane attack complex and hence complement dependant cell lysis, or macrophage activation.

TABLE OF CONTENTS

List of Tables

List of Figures

Abbreviations

ab	antibody
ag	antigen
bc	bile canaliculus
C	degrees centigrade
CD	conjugated dienes
CPM	counts per minute
DFO	deferoxamine (or desferrioxamine)
EM	transmission electron microscopy
FSC	fat storing or Ito cell
g	gram
HPF	high power field
IC	immune complex
ip	intraperitoneal
IF	immunofluorescent
KC	Kupffer cell
L	lymphocyte
LSFP	lipid soluble fluorescence products
MAC 1+	cells positive for Mac 1+ (monocyte/macrophage) antigen
mPCA	monocyte procoagulant activity
N	neutrophil
NS	not significant ($p > .05$)
OR	oxyradical
PBS	phosphate buffered saline
pi	post infection
PMN	polymorphonuclear leukocyte
PFU	plaque forming unit
SH	SH groups on protien
TBA	thiobarbituric acid
TPV	terminal portal vein
RBC	red blood cell, erthryocyte
SD	standard deviation
SE	standard error of the mean
SEM	scanning electron microscopy
TcR	T cell receptor
ThV	terminal hepatic venule
TEM	transmission electron microscopy
wt	weight
Z1, Z2, Z3	zones 1,2,3 of the liver acinus, Z1 = periportal, Z2 is intermidate between Z1 and Z3, Z3 = near the ThV.

INTRODUCTION

Historical Review

Human Hepatitis Despite various historical references to jaundice since the time of Hippocrates, an infectious origin for hepatitis has only been proposed since the early 20th century, by Botkin, Heitler, and Flindt (Mosley and Galambos 1969). However, the view that jaundice was a disease of the biliary passages prevailed even into the 1940's. Strong doubt was cast on this view when the incidence of post-vaccination jaundice increased to the point that an infectious cause had to be considered. Simultaneously, it was noted that there was a high incidence of jaundice in diabetic clinics, and after gold therapy, in arthritis clinics. The introduction of pooled plasma for transfusions during World War II also led to outbreaks of epidemic jaundice (Sartwell 1947). As a result, the urgent need for manpower led to an intensification of the search for an infectious agent. Various etiologies have been suggested and discarded: a bacterial cause by Sarrailhe in 1916, or intoxication by Eppinger (Mosley and Galambos 1969). Viruses were first suggested in the 1920's, but it was not until the 1940's that British and American groups demonstrated the presence of two virus-like agents. MacCallum first named them in 1947, with the result that during the 1950's they commonly began to be distinguished by the terms Hepatitis A virus (HAV) and Hepatitis B virus (HBV) (Mosley and Galambos 1969).

In 1964 B.S. Blumberg discovered the Australia Antigen which was later recognized as a marker of serum hepatitis (Blumberg et al 1967). He received the Nobel Prize in 1976 in recognition of his work with HBsAg. Hepatitis B is a 42 nm DNA virus (with 4 known subtypes) in the family Hepadnaviridae, which also includes some species-specific

1

animal hepatitis viruses (e.g. Woodchuck hepatitis virus). HAV was identified as a 27nm naked RNA virus in the family Picornaviridae by Feinstone et al (1973). The human strain was identified as CR326 (Provost et al 1975).

In the 1970's it became clear that another virus must be involved in certain cases of human hepatitis. In 1977 Rizzetto reported a virus with a small circular RNA genome, delta virus (HDV), which was only found in the presence of HBV. This virus has a relatively simple genomic structure, similar to that seen in plant viroids, i.e. both are single stranded RNA similar to introns (Lewin 1986). With the establishment of the roles of HAV, HBV, and then HDV, it became apparent that these three did not account for all the known cases of hepatitis. To this date, the remainder are still referred to as Non-A, Non-B (NANB) viruses. They can be divided into two groups: those transmitted via fecal-oral spread, and a second group primarily parenterally transmitted. Both long and short incubation forms of both types of NANB viruses are known.

The enterically transmitted type of NANB is exemplified by the waterborne epidemics of NANB seen in India. A 22-27 nm spherical virus has been isolated from stool extracts from these patients. An enzyme linked immuno assay (ELIZA) for enteric NANB has been developed which does not cross-react with HAV (Gupta et al 1988). Similarly, epidemics due to water contamination with fecal material have been seen in Algeria, Nepal, Burma, Pakistan, Ivory Coast, Somalia, and Mexico (Belabbes 1985). The viruses from all of these epidemics were found to be between 27-34 nm (Bradly et al 1988), and serum samples from these epidemics showed cross reactivity. Viral isolates from these epidemics have been transmitted to cynomolgus macaques (Bradly et al 1988, Krauczynski 1989). NANB hepatitis has also been experimentally transmitted from

humans to monkeys via fecal extracts (Balayan 1983, Favorov et al 1986, Andzhaparidze 1986, Zairov 1984).

The virus causing one of the parenterally transmitted forms of NANB, known as strain H, is between 30-60 nm, with an unknown genome, a lipid coat, and has been transmitted to chimpanzees (He et al 1987). None of these viruses has been fully characterized in a widely accepted manner. Another, possibly parenterally transmitted NANB agent, has been provisionally named GB agent, after the Chicago surgeon from whom it was isolated (Karayiannis, 1989). The GB agent has been reported to be between 20-36 nm, ether resistant, and able to induce disease in tamarins (Karayiannis, 1989). Molecular hybridization studies have shown that the GB agent has no genetic relationship with hepatitis A. It has even been proposed that one of the NANB hepatitis agents might be a prion, on the basis that a causative agent was found which was resistant to heat and alcohol treatments that are known to inactivate HTLV-III (Kingdon 1987). Another theory is that one agent is a spuma virus. The latter has been discounted by Williams (1988), on the basis that there is no correlation between their presence and pathological changes.

Other less clinically important viruses have been noted to induce similar types of liver injury. Adenovirus-type 2 causes numerous sharply circumscribed zones of coagulation necrosis in Swiss mice with thymic alymphoplasia. Several strains of adenovirus have also been isolated from patients with viral hepatitis but their significance is uncertain. Cytomegalovirus (CMV) infection also leads to numerous characteristic foci of hepatocellular necrosis, as do coxsackie B , herpes simplex, Epstein Barr, reovirus and rubella viruses. Similar lesions are found in other organs indicating that the hepatic

changes are only one facet of a generalized reaction of the entire reticuloendothelial system (Klatskin 1969). In animals, other hepadna viruses such as woodchuck hepatitis virus (WHV) (Venkateswaran et al 1987), ground squirrel hepatitis virus and Peking Duck hepatitis virus all induce similar lesions.

Much work has also been done in describing the liver pathology, clinical patterns and alterations in liver function tests occuring during the various viral hepatides. In contrast, investigation into the mechanisms by which these viruses induce disease have been recent, sporadic and stem mainly from the recent rapid explosion of knowledge in the area of immunobiology and especially autoimmunity. Such principles have been helpful in developing an understanding of the mechanisms behind viral persistence and the various manifestations of chronic hepatitis. However, chronicity is a feature primarily of HBV and NANB infections where, even there, it occurs in only a minority of cases. The most pressing question concerns the mechanism of induction of acute hepatocellular necrosis, and particularly the factors that lead to fulminant hepatic necrosis, which are still not well understood (Yoshida et al 1987).

Although incubation times, routes of transmission, and tendencies to persistence vary, the nature of the initial lesions in acute viral hepatitis are indistinguishable histologically and ultrastructurally (Phillips et al 1987b) whether the virus is HAV, HBV, NANB, a species specific hepadna virus or one of the murine hepatitis viruses (MHV). The usual observation is that of sharply circumscribed (focal) eosinophilic (or 'coagulative ') necrosis of hepatocytes with a sparse mononuclear cell infiltrate, occasional polymorphonuclear leukocytes (PMN's) and rare eosinophils. Descriptions often refer to

Kupffer cell alterations and proliferation and to the appearance of acidophilic bodies. In the case of fulminant hepatitis, these lesions progress to near confluence at the time of death. Ultrastructural studies have shown no significant difference in lymphocytic interaction with hepatocytes in patients with HAV, HBV or NANB hepatitis (Phillips et al 1987b). In certain strains of mice (eg.C3HebFe/J) and certain individuals, the initial lesions are followed by the development of a significant PMN and lymphocytic infiltrate culminating either in resolution or development of a granulomatous hepatitis (MacPhee et al 1985).

In the 1960's the similarity of the MHV-3 induced lesions in mice to those seen in human viral hepatitis led to the use of this mouse model for further investigations. The availability of genetically defined inbred strains made it possible to reliably reproduce an acute, chronic, fulminant or fully resistant hepatitis after infection with MHV. Murine hepatitis virus type-3 is one of the hepatotrophic strains of MHV that has been used by several groups to investigate the pathogenesis of viral hepatitis in mice. In addition, a rat strain (LEC) with an autosomal recessive mutation leading to spontaneous onset of hepatitis at 3-4 months of age has recently been described (Yoshida et al 1987). The main histological change in LEC hepatitis is focal hepatocellular necrosis without inflammation. About 80-90% of the rats that survive for over a year develop hepatocellular tumors.

MHV-3 Hepatitis MHV-3 was first isolated by Dick, Niven and Gledhill (Dick 1955) from Swiss mice. This virus was identified as one of the coronaviridae on the basis of that group's distinctive morphology on negative stains: all members have large glycoprotein peplomers projecting from the envelope. It is a single-stranded, positive

polarity RNA virus. The genomic RNA is surrounded by a lipid bilayer envelope with two structural proteins called E1 and E2 (Holmes 1985). Most coronaviruses are trophic for epithelial cells (e.g. respiratory or enteric tracts), however, the reason for their restriction to replication in these cells is unknown (Holmes 1985).

The organotrophism and virulence of MHV-3 varies with the age and the genetic background of the host. MHV-3 is considered one of the most virulent strains of MHV for adult mice. Only A/J mice are fully resistant and exhibit no clinical or pathological evidence of disease. DDD mice have a clinically inapparent hepatitis and, like the A/J strain, clear the virus in 7 days. Some variants of C3H mice develop an acute hepatitis and recover fully, while other individuals go on to develop chronic aggressive or granulomatous hepatitis and die prematurely (Wege et al 1982, MacPhee et al 1985). Balb/cJ and C57BL6/J mice are both fully susceptible and die of fulminant hepatitis within 4-7 days of infection.

MHV-3 was first noted to selectively destroy macrophages by Bang and Warwick (1960) using an in-vitro macrophage culture system. This was a modification of their liver fragment explant culture system, and used an MHV-2 (Princeton) strain of virus and the susceptible Princeton strain swiss mice. C3H (Andervont) mice are clinically resistant to MHV-2. However, their macrophages did show evidence of degeneration and granularity but did not disintegrate as did those from susceptible mice. They also observed that susceptibility is inherited and that susceptibility and resistance can be segregated in the F2 and backcross generations (Bang and Warwick 1960). Using mixed cultures of macrophages they showed that the factors for resistance or susceptibility were in the cells, and were not related to a humoral factor.

An electron microscopic study of liver damage induced by the then newly discovered MHV-3 strain was reported in 1962 (Svoboda et al 1962), where the effect of MHV-3 was compared with MHV-2 in CFW mice. They confirmed the finding of early lysis of the Kupffer cells followed by focal coagulative necrosis of hepatocytes. They declared there was no ultrastructural difference between the lesions induced by these two stains of MHV, and the findings were reported together.

Current Understanding of Liver Anatomy The location of the liver between the gut and the systemic circulation is significant in the host response to many foreign pathogens, including viruses. Blood enters the liver via the portal vein and the hepatic artery. Both afferent channels flow in the same connective tissue tracts, along with the biliary tree, until they reach the finest terminal branches of the portal vein (TPV's). At this point the arterioles empty into the sinusoids near the TPV's, causing some sinusoids to have higher flow rates and to be intermittently arterialized. Due to interconnections between sinusoids, the blood is well mixed by the time it crosses the sinusoidal bed and reaches the smallest efferent vessels, the terminal hepatic veins (ThV's).

The area of parenchyma supplied by one TPV is a functional unit known as the liver acinus. The acinus may be divided into three zones (Z_1, Z_2, Z_3). The area closest to the TPV is zone 1, zone 2 is the intermediate area and zone 3 is closest to the ThV's (Rappaport 1976). The sinusoids run between the cords of hepatocytes, which are never more than 1-2 cells thick. Each hepatocyte therefore has one or more surfaces exposed to the blood.

In the murine liver the sinusoids are lined by endothelial cells which appeared to have gaps between them, as well as numerous fenestrae. Murine hepatic sinusoidal endothelial

cells have properties intermediate between those of human endothelial and Kupffer cells. More than half of the endothelial cells are peroxidase positive along the nuclear envelope and the endoplasmic reticulum (Stohr 1978), a property which in humans and rats is characteristic of Kupffer cells. Endothelial cells normally take up small particles (Steffan et al 1986) 3 - 6 times better than Kupffer cells (Fahimi 1982), and are important in the uptake of macromolecules from the blood via clathrin coated pits. In mice, however, endothelial cells have been seen to readily take up latex particles of 0.2 micron diameter, and occasionally to take up 2.0 micron latex beads (Yamashita et al 1985). Kupffer cells, especially in Zone 1, on the other hand, are better at phagocytosing large particles. When carbon particles are injected i.v. in the mouse, within minutes the entire endothelial surface is covered with scattered particles. However, within 24 hours all particles will be found concentrated in a localized area within Kupffer cells. In addition to phagocytosis, murine endothelial cells have other properties similar to Kupffer cells: they can also take up and present antigen, and hence have a role in the immune response (Wisse et al 1989).

Lying under the sinusoidal cells are the fat storing (FSC) or Ito cells. They can be identified by characteristic lipid vacuoles rich in Vitamin A. FSC are believed to be responsible for fibrogenesis (Fahimi 1982), as they are frequently observed in association with collagen fibrils. In addition, contractile pericytes have been described, but now are considered identical to FSC (Wake 1989).

Another type of cell observed in the liver is the Pit cell (Wisse et al 1976), so called because of its small and very electron dense granules which contain neuroendocrine-like materials (Bouwens et al 1987). It has been proposed that Pit cells have natural killer (NK) cell activity, and may represent a resident liver NK cell. It appears that Pit cells

seen in the liver by electron microscopy are the same cells as the natural killer cells of tumor biology, and the large granular lymphocytes (LGL) of immunological or hematological assays (Wisse et al 1989). In rats, these LGL are either asialo-Gm-1 positive or negative (Bouwens et al 1987). The asialo Gm-1⁻ cells have antibody dependant cell mediated cytoxic (ADCC) activity and the asialo-Gm-1⁺ cells have NK activity. Pit cells are also found in human peripheral blood (Bouwens and Wisse 1989), although they are somewhat larger there. In both humans and rats they are found in very low numbers, i.e. about 10 cells per mm^2 of liver tisue (Bouwens and Wisse 1989). Enzan et al (1989) have also demonstrated the presence of Pit cells by immunoelectron microscopy in both rat and mouse livers. They found that these cells were even more scarce in mice (eg. ICR) than in rats (eg. Wisser strain).

T and B cells have also been shown to exist in the liver. T-cells are said to represent about 1 - 2% of the non-parenchymal liver cells in the rat and B cells about .01% (Malter et al 1986). It is questionable whether T or B cells are permanent residents in murine liver.

The Role of Liver Sinusoidal Cells   The origin and precursors of all the non-parenchymal liver cells remains a subject of debate (Jones and Summerfield 1982, Bouwens et al 1986, Naito et al 1986). The controversy over whether Kupffer cells are constantly being replaced by the bone marrow or are self-renewed seems to be becoming clearer, as both self-renewal and replacement with new precursors from the marrow have been demonstrated. At a recent symposium (Wisse et al 1989) it was concluded that the evidence is in favor of the existence of two types of macrophages in the liver: resident Kupffer cells and recently immigrated monocytic cells. Both macrophage types can

proliferate in the sinusoids and migrate in and out of the liver (Wisse et al 1989). Under certain conditions i.e. partial hepatectomy or toxic injury, one or the other macrophage type may be the primary source of new liver macrophages. Diesselhoff-Den Dulk et al (1979), using tritiated thymidine as a marker, concluded that during an acute inflammatory response (induced by stilboestrol, zymosan or corynebacterium), the increase in liver macrophages was due to an influx of labeled precusors from the bone marrow. Some evidence exists that Kupffer cells can both replicate in-situ under certain stimulatory conditions (Decker et al 1985) and are continually being renewed by precursors from the bone marrow (Bouwens 1988 and Geerts et al 1988).

Kupffer cells have traditionally been considered fixed tissue macrophages, as emphasized in the statement that "Kupffer cells don't move. Or if they do we can't see them" (Carr 1977). However, studies using carbon or iron labelled Kupffer cells during CCL4-induced liver necrosis have demonstrated migration of Kupffer cells from the undamaged (Z_3) areas of the liver to the necrotic periportal areas (Parry 1978). Hardonk et al (1986) demonstrated that carbon labelled cells migrated first to the portal area and then to regional lymph nodes. No migration route for these Kupffer cells was suggested except for the comment that cells seen in the portal tracts 'may originate from blood or liver'. This thesis presents evidence that certain large cells can migrate upstream. Their size and morphology, as well as migratory habits suggest these cells are liver macrophages. Similar cells have also been observed in the spleens of rats and mice (Schmidt et al in press).

Most of the secretory products known to be produced by macrophages (now numbering more than 80) have been confirmed for Kupffer cells. These include: fibronectin (Reider

et al 1982, and Vincent et al 1989), interferon alpha and beta (Kim et al 1982a, Zenilman

et al 1988 and Werner-Wasik et al 1989), procoagulant activity (Maier and Hahnel 1984),

prostaglandins D_2, E_2, PGF_2alpha, 6 keto PGF_1alpha (Bowers et al 1985, Rieder et al

1988, Karck et al 1988, Shirahama et al 1988a, Kuiper et al 1988a,b, Dieter et al

1986,1987a Ouwendijk et al 1988, Casteleijn et al 1988, Brower et al 1988),

thromboxanes including TxB_2 (Brower et al 1988), leukotrienes including LTE_2

(Sakagami et al 1988, Hagmann et al 1987), excited oxygen radicals (Virk et al 1988,

Filice 1988) including superoxide (Reider et al 1988a,b), hydrogen peroxide (Murry and

Nathan 1988), chemiluminescence (Peterhans 1979, 1980, Virk et al 1988) interleukin-1

and interleukin-1 inhibitor (Shirahama et al 1988b), hepatocyte stimulating factor (HSF)

(Kurokawa et al 1988), tumor necrosis factor (Karck et al 1988, Decker et al 1989), TNF

alpha (Magilavy and Rothstein 1988), cathepsins B and H (Yokota and Kato 1987),

complement components (Prasad et al 1987), and erythropoietin (Paul et al 1984), among

many others.

The normal function of Kupffer cells in vivo seems to be the routine phagocytosis of

senescent red cells with subsequent recycling of iron in the form of ferritin, and the

degradation of bacteria, viruses and other foreign particulate matter (Wardle 1987,

McCuskey et al 1987). Kupffer cells also play a role in lipid metabolism as indicated by

the presence of receptors for low density lipoproteins on their surface (Wardle 1987)

Stimulated Kupffer cells induce proliferation of FSC cells (Zerbe and Gressner, 1988)

and FSC have been implicated in fibrogenesis. In some cases Kupffer cells may also take

up platelets (Neiman et al 1987). Kupffer cells can process and present antigen, and are

known to express HLA.DR (human) or Ia (mice) antigens (Dixon et al 1986). As antigen

presenting cells they form an important link in the host immune response to foreign pathogens, many of which enter the circulation via the gut and are removed by first passing through the liver (Marshall et al 1987). Kupffer cells have numerous receptors which aid in this process, for example Fc receptors (FcR) for IgE (Muro et al 1987,1988) and for IgA (Sancho et al 1986). As previously mentioned, there are special receptors for LDL, glucagon (Watanabe et al 1988), mannose (Zenilman et al 1988) and for C3b (Ding and Nathan 1988, Steffan and Kirn 1986). The products of activated Kupffer cells also have a significant role in regulating hepatocyte function. This occurs primarily through PGD_2, the primary prostanoid produced by Kupffer cells, which induces glucose release from hepatocytes.

Sinusoidal cells in viral infections   Kupffer cells have been recognised as an important part of the host defense system against viruses (reviewed by Latham 1988), along with the whole monocytic lineage and the other organ specific members of the reticuloendothelial system . Various methods for depletion of mononuclear phagocytes have indicated their significance in non-specific host resistance . Macrophages can extrinsically inactivate or inhibit certain viruses (HSV, CMV, Marek's, MHV, ectromelia) via complement or lymphokines, by amplifying specific T-cell immunity, via antibody mediated cellular (macrophage) cytotoxicity for virally infected cells, or by excluding viral uptake and multiplication. Intrinsic mechanisms include interference in viral replication or release, induction of latency, or transformation of the virus. In addition macrophages can kill viruses via products of oxidative metabolism and possibly by enzymes, and can block replication via interferon (Morahan et al 1985, Stohlman

1983). In both cases the mechanism is virus specific as well as species specific. This makes it difficult to extrapolate results from one virus or animal species to another.

Viruses may also directly alter macrophage functions, and here again the effects are specific to the viruses. Many depress normal macrophage functions, while others have either no effect or an enhancing effect. For example, macrophage infection with certain viruses (eg CMV, influenza, MHV, Sendai, and polioviruses) can suppress lymphocyte function. Experiments using activated murine macrophages have shown that the release of PGF_2 and H_2O_2 may be responsible for this suppression of lymphocyte proliferation (Metzger et al 1980).

Viruses can also stimulate release of reactive oxygen species from phagocytic cells. Certain viruses (eg Sendai, parainfluenza-3, Newcastle disease virus, influenza, and poxvirus) can stimulate cells directly (Peterhans 1987). Most other viruses activate phagocytic cells only when bound to antiviral antibodies (Peterhans et al 1988).

This extremely limited current understanding (Lever 1987) of the mechanisms of acute viral injury has resulted in the situation, true to the present, where there is no specific treatment available for acute viral hepatitis. Further, as effective intervention is unlikely to become known available (outside of fortuitous discovery) without a rational understanding of how viruses induce hepatocellular necrosis.

This thesis presents evidence for the hypothesis that host-determined factors, as opposed to direct effects of a hepatitis virus are responsible for the induction of fulminant hepatocellular necrosis by MHV-3. The nature of the lesions was examined using light, electron, scanning and in vivo microscopy. As T-cells have previously been found to be

responsible for host directed attacks on virally infected cells, immunofluorescent tagged antibodies to cell surface markers were used to define the cell types associated with the developing areas of necrosis. In vitro studies were performed to determine whether strain dependant differences exist in the function of lymphocytes and macrophages. Serum was tested for antibody to MHV both pre- and post-infection. Biochemical studies were employed to determine whether free radical reaction products developed after infection, and whether the formation of these products could be abrogated using free radical scavengers or other inhibitors.

MATERIALS AND METHODS

Animal Model

Five week old male mice of the Balbc/J (H-2d), A/J (H-2a) and C3HebFe/J (H-2k) strains were obtained from Jackson Laboratories, Bar Harbour, Maine, and housed in microisolator units. These consist of plastic cages holding up to five mice, with individual HEPA filter removable tops. Mice were maintained on Purina Lab Chow ad libitum and were infected with MHV-3 between the ages of 6-8 weeks. Animals were ether anesthetized, organs removed, then sacrificed by cervical dislocation at 12, 24, 48, 72 or 120 hours after infection. Control mice were not deliberately infected with virus.

Virus

Murine hepatitis virus Type 3 (MHV-3) was obtained from the American Type Culture Collection, propagated in L-2 cells (Wilson et al 1986), and harvested when 50% of a monolayer of infected cells had lifted off the plate. Cells were disrupted by passage through a 21 gauge needle and cell debris was removed by low speed centrifugation (250 g for 10 min). Aliquots of the supernatant were stored at -70C, and infectious virus assayed by plaque formation on L-2 cells (Levy et al 1981). The LD_{50} was determined by using two trials with different lots of MHV-3. In each experiment three groups of five mice (total n = 30) were given 0, 1 and 10 PFU's of MHV-3 intraperitoneally and time to death determined. The LD_{50} was found to be 5 PFU, with a survival time of 5-7 days. When inactivated virus was required, it was prepared by exposure to an ultraviolet (full spectrum) light source at a distance of 7 cm for 5 min, with the temperature controlled by means of an ice bath. Effectiveness of the inactivation was monitored by retitering the virus to determine the least amount of irradation necessary to decrease viability. The plate was kept on ice to prevent denaturation of the viral proteins.

Monocyte Chemiluminescence

Spleens were removed from groups of ether anesthetized mice prior to, and at 48 hours following MHV-3 infection. Spleens were expanded with an injection of cold RPMI to facilitate separation of cells, then punctured with a needle several times and gently squeezed with forceps until only an empty sac remained. Mononuclear cells were separated on Ficoll-Hypaque (specific density 1.077) and resuspended in phosphate buffered saline (PBS) at pH 7.2. Cells were counted in a hemocytometer and viability assessed by trypan blue exclusion. Viability was always greater than 99%. The monocyte content was determined by non-specific esterase staining according to the method described by Lam and Switzer (1971). An aliquot containing 10^5 monocytes was placed in scintillation vials (Wheaton Glass Ltd., Listowel, Ontario and New Jersey).

Chemiluminescence was amplified by the addition of 50 ul luminol (5-amino-2, 3-dihydro 1, 4-phthalazinedione, Sigma Chemical Co., St. Louis, Mo.). Luminol was dissolved in 4 mg/ml dimethylsulphoxide (DMSO), made up fresh and kept in the dark. Vials were then counted in an in-coincidence mode in a Scintillation Counter (Model LS230, Beckman Scientific) to obtain pre-stimulation levels of chemiluminescence. Following this, 0.4 ml of opsonized zymosan in PBS was added to each vial and counted for one minute. Opsonized zymosan (Sigma) was prepared by incubation at 37 C for 30 minutes at a concentration of 16 mg zymosan per ml serum, which was obtained from the same mouse as the spleen cells being tested. Post-incubation, the zymosan was pelleted by centrifugation (250 g x 5 minutes) and then resuspended in PBS to a final concentration of 10 mg/ml.

Measurement of Free Radical Reaction Products

One lobe of the liver was homogenized in an approximately 10% liver/sterile water suspension. Protein determinations were done on each liver homogenate using the Biorad Micro-Assay Kit, as described by the manufacturer (Biorad Protein Assay Kit, Biorad Laboratories, Richmond, Cal.) and all reaction products were calculated per mg protein.

a) Conjugated Diene Assay

Three hundred ul of a 10% liver homogenate was added to 10 ml of 50:50 chloroform:methanol and incubated for one hour at room temperature under nitrogen (N_2) to prevent oxidation. After this, 25 mls of H_2O was added, then centrifuged for 10 minutes at 250 g. The water layer was discarded and 5 ml fresh H_2O added and respun. The chloroform layer was evaporated under N_2 and the residue resuspended in 0.5 ml fresh chloroform. A 400 ul aliquot of the above extract was again evaporated under N_2, and then resuspended in 1 ml of cyclohexane. The absorbance was read at 232 nm and conjugated dienes were calculated per mg protein.

b) Lipid Soluble Fluorescent Products

A 100 ul aliquot of the final chloroform extract from the conjugated diene assay (above) was added to 0.9 ml of 9:1 cholorform:methanol. Fluorescence was measured at 340 nm excitation and 430 nm emission in a Perkin-Elmer Model 650-15 Fluorescence Spectrophotometer. The positive control was 1 ug/ml quinine SO_4 in 0.1 N H_2SO_4, a 100 ul sample of which, treated as above, gave 470 Fluorescence units.

c) Thiobarbituric acid Assay

Liver homogenate (50ul) was incubated with 1.5 ml 20% acetic acid, 0.2 ml 8.1% fresh SDS and 1.5 ml of 0.8% TBA for one hour in a 95 C water bath which was covered to prevent evaporation. Then 5.0 ml of 15:1 v/v N-butanol: pyridine (Sigma) were added,

and centrifuged at 1,000 g for 10 min in closed polypropylene tubes. The absorbance of the supernatants was read at a wave length of 532 nm in a spectrophotometer (LKB Biochrom Ultrospec Pi) against 0, 10, 20, 30 and 40 nM standards of malonaldehyde (MDA) (1,1,3,3 tetramethyl propane, Sigma) in water. The amount of MDA in each sample was determined by interpolation from the standard curve.

d) Measurement of SH groups

A 50ul aliquot of 10% liver homogenate plus 1.5 ml of 0.1 M KPO_4 pH 8.0 [containing 0.67 mM 5,5 dithiobis-2 nitro benzoic acid (DTNB)] was mixed and incubated for 10 min. at room temperature, then spun at 2600 g for five minutes in an Eppendorf Microfuge. The absorbance of the supernatant was read at 412 nM. The control was the same without the DTNB.

Oxyradical Scavenger Studies

A potent experimental inhibitor of lipid peroxidation (Lazeroid U74006F) was kindly supplied by Dr. M. Braughler, UpJohn Scientific, Michigan and was administered at 1,10 and 100 mg/kg/bid orally by intubation. The 1mg/kg/bid dose was chosen for the scavenger studies after pilot studies showed that this was the best inhibitor of conjugated diene formation. Deferoxamine (DFO) was obtained from Ciba-Geigy, Canada Ltd. (Mississauga, Ont.). It was dissolved in sterile saline and administered by continuous subcutaneous infusion at 4.2 mg/day, starting just prior to infection with MHV-3, using an Alzet mini-osmotic pump (Model 2001, Alza Corp., Cal, USA), which was implanted under the skin on the upper backs of the mice. Nicotinamide (C.E. Jamieson & Co. Ltd., Windsor, Ont.) was dissolved in 0.2 ml normal saline at a dose of 10 mg/mouse and given by i.p. injection once daily. Superoxide dismutase and catalase were obtained from Sigma Chemicals, St Louis, Mo., and administered at 8mg/kg twice daily in 0.1 ml of saline. N,N 1-diphenyl-p-phenyline diamine (DPPD), a well known inhibitor of lipid

peroxidation, was given at a dose of 600 mg/kg in 0.1 ml of olive oil i.p. twice a day. In studies by Quanguan and Moldeus (1988), this dose was able to inhibit bromobenzene toxicity to hepatocytes. DPPD was a gift from the laboratory of Dr. A Autor, Pulmonary Research Laboratory, St Paul`s Hospital, Vancouver , B.C. CV3998, a platelet activating factor antagonist, was given in 0.1 ml saline at a dose of 10 mg/kg i.p. twice daily, as found effective in a shock model in rats (Toth and Mikulaschek 1986); it was kindly supplied by Dr. H. Salari, Dept of Medicine , University of British Columbia, B.C.

Cyclosporine administration

Cyclosporine was obtained from Sandoz Ltd, Basle, as a suspension in olive oil. It was administered twice daily by oral gavage at a dose of 70 mg/kg/day from the time of infection until sacrifice or death. Serum levels of CsA were measured by RIA using a polyclonal antibody kit (Sandoz Ltd, Basle), to assure adequate therapeutic concentrations. The doses were chosen to reflect the best possible balance between immunosuppression and toxicity, according to work done previously by Dr Keown's laboratory (Keown PA, Stiller CR, McKenzie N, and Blackler K. Comparison of cyclosporine and Nor-val cyclosporine in experimental autoimmune and alloimmune injury. Keown PA, Stiller CR and Blackler K. Effect of cycloporine in murine autoimmune hemolytic anemia:Inhibition of anti-erythrocyte antibody production and preservation of suppressor cell generation. Manuscripts in preparation.)

Light and Immunofluorescent Microscopy

One lobe of the liver was removed at sacrifice and paraffin embedded for light microscopy. Four micron sections were routinely stained with hematoxylin, phloxine and safranine (this combination of stains will accentuate any hepatic fibrosis that exists). The extent of necrosis was assessed using either a hundred square microscope grid and averaging three fields per mouse, or using a Kontron Image Analysis System (Kontron

Bilanalyze, Munich), in which the necrotic area was computer defined and two fields on each of three biopsies per mouse were analyzed. In both cases means and standard deviations of the area of necrotic cells were calculated per mouse, and then for each treatment group. The use of at least a hundred square grid over random sections of a low power field ensures representative sampling (Trump et al 1965).

The remaining lobe was quick frozen (using liquid nitrogen) for immunofluorescent microscopy. Sections (4um) were fixed for two minutes in 100% acetone at 4 C, air dried for one hour at room temperature (or overnight at 4C), then rehydrated and blocked by a 30 minute wash with 10% normal serum (10% rabbit serum, 10% normal mouse serum in PBS pH 7.2). Rat monoclonal antibodies to murine cell surface antigens were obtained from Sera-Lab (Dimension Laboratories In., Mississauga, Ontario), and included anti-Thy-1, (MAS 108C, dilution 1/100), anti-Lyt-2 (MAS 111C, dilution 1/10), anti-L3T4, (MAS 110, dilution 1/10), anti-Mac-1 (MAS 034, dilution 1/50), anti-IA (MAS 053C, dilution 1/10), and anti-IgG (Zymed, dilution 1/40). Sections were incubated with primary antibody (in 10% normal serum/PBS) for 30 minutes, washed with PBS, and treated with the second antibody, FITC labelled rabbit anti-rat immunoglobulin, rinsed with distilled water, and air-dried and mounted in buffered glycerine pH 7.2 (50/50 glycerine/PBS). Slides were photographed using a Zeiss fluorescent microscope and Zeiss 35 mm camera. The number of positive cells could then be counted per field (10, 20 or 40 X). All anitbodies were tested on normal murine spleen cells from the same strains of mice and stained the expected number of cells.

In Vitro Lymphocyte Proliferation

Peritoneal macrophages were collected from ether anaesthetized mice by intraperitoneal injection of 5 mls of cold RPMI 1640 cell culture medium (Gibco Laboratories) using a 23 gauge needle, gentle abdominal massage and withdrawal of fluid. The cell suspension

was washed twice with cold RPMI at 1,000 rpm for 10 min. The cells were counted and the percentage of monocytes determined by non-specific esterase staining. The cell suspension was plated at a concentration sufficient to ensure 1×10^5 monocytes per well of a 96 well round bottom plate (Titretek Company) and incubated for 1-2 hours at 37 C to facilitate attachment. Plates were then emptied to remove non-adherent cells and fluid. Virus (10^4 PFU) in RPMI or RPMI alone was added to the wells and incubated for 30 minutes while rocking at room temperature. Unattached virus was then removed by washing twice with 150 ul warm RPMI, followed by a 10 minute centrifugation at 1,000 rpm , and discarding of supernatant.

Splenic lymphocytes were obtained by gentle teasing of the spleen in RPMI with forceps, until only the connective tissue remained intact. Mononuclear cells were separated by centrifugation over Ficoll-Hypaque (specific density 1.077), and were then washed twice with RPMI before counting. Splenic lymphocytes (2×10^5 in RPMI with 10% fetal calf serum) were added to the macrophages in the microtitre wells. The plates were then incubated for 48 hours (time selected was based on previous studies by C.Strejan, personal communication) at 37 C in 5% CO_2, followed by an additional 18 hours with ^3H thymidine (0.5 uCi); and then washed twice again with warm RPMI. The wells were harvested and counted in a liquid scintillation counter (Beckman Scientific Co.).

Transmission Electron Microscopy

Mice were ether anesthetized, a laparotomy performed, and the entire liver removed. One lobe was immediately placed in chilled (4 C) glutaraldehyde buffer, and several wedges were removed and minced while still in the buffer. The specimens were then placed in fresh solution and fixed by immersion in a 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer for a minimum of 24 hours at 4 C. The material was then washed in

buffer, cut into 0.5 mm cubes and osmicated for 45 minutes. Tissues were stained en bloc with uranyl citrate and lead acetate, and then dehydrated through a graded alcohol series, followed by acetone, graded Epon and finally embedded in 100% Epon. Ultrathin sections were cut with a diamond knife and viewed in a Zeiss 109 electron microscope at 50 Kvolts.

Microcorrosion Casts

In preparation for corrosion casting of the liver, the abdominal aorta was cannulated and heparin (50 USP/100g body weight) was administered to prevent clotting. The body was perfused with Ringer's solution at 100 cm H_2O pressure for 20 min or until liver was blanched, the vena cava having been cut for drainage. At this time a modified, low viscosity Batson's casting compound (Nopanitaya et al 1979) was injected manually via the arterial cannula. The liver was left undisturbed for 1 1/2 hours during polymerization; it was then cut into several pieces and the tissue digested with 40% KOH at 60 C for 3-4 days. The cast was then rinsed in distilled water, air dried, mounted on SEM stubs and sputter coated with gold. The casts were examined using a Phillips 501 scanning electron microscope.

In Vivo Microscopy

Mice were injected with MHV-3 (10^4 PFU i.p.) and examined at 24-48 hr post-infection (p.i.). Anesthesia was induced with sodium pentobarbital, 6 mg/100gm body wt. A midline abdominal incision was made and the mouse was placed on its side on the platform of an inverted microscope, so that one lobe of the liver lay on a window positioned over the objective lens. The liver was moistened continuously by a saline drip (37 C), and the animal's rectal temperature was monitored and maintained at 37 C with a heat lamp. The liver was covered with Saran wrap which held it gently against the coverslip window, restricting lateral and vertical motion (due to respiration and heart

beat) at the plane of focus. Illumination was provided by a Volpi HL250 fiber optic source positioned obliquely relative to the liver, which resulted in improved image contrast. Objective lenses ranging in power from 10-100 x (oil immersion) were used. A Panasonic video camera (WV-1550, Newvicon tube with extended red sensitivity) was mounted over the inverted microscope and the image was displayed on an Electrohome monitor. A Panasonic WJ-810 character generator added stopwatch information to the videosignal, which was recorded using a Panasonic NV-924XD videocassette recorder. This technique has been described in detail recently (MacDonald et al 1987).

Radioimmune assay (RIA)

In order to screen for the presence of antibody to MHV-3, 96 well plates (Flow Laboratories Inc, Mississauga, Ont) were coated with MHV-3 (grown in L-cells), 20 ug protein per well (i.e. 10^5- 10^6 PFU /well) in 1 ml of RPMI. The wells were incubated overnight at 4C and then allowed to dry at room temperature. The plates were washed 5 times with PBS (pH 7.4) and then incubated with a blocker of 1% fetal calf serum or 0.5% bovine serum albumin (BSA) rather than normal mouse serum for 15 minutes, as prelimary trials and other workers have shown that it is very common for normal laboratory mice to be antibody positive for MHV (Lussier 1986, Talbot 1985, Fujiwara 1971). After washing 5 times with PBS-Tween 20 (0.5%), the wells were incubated with test serum (neat, 1:5, 1:10 dilutions) for 2 hours at room temperature. This was followed by washing 5 times with PBS-Tween 20. The linker, rabbit antimouse Ig (100 ul of a 150ul stock in 10 ml PBS) was then incubated with ^{125}I-protein A (30,000 counts/well in 10 ul) for 2 hours at room temperature. Wells were then washed an additional 5 times with PBS-Tween 20 and allowed to dry. The wells were cut out and counted the same day in a gamma counter. Cell lysate without virus and medium alone (background) were

used as negative controls. Antibody to JHMV nucleocapsid is cross-reactive with MHV-3 nucleocapsid (Talbot et al 1984).

Statistics

Data was tested for significance using the two sample t test for independant samples with equal (pooled) variances according to Rosner (1986).

RESULTS

Microcirculatory Studies

### a) Normal liver microcirculation of the Balbc/J mouse.

In vivo microcirculatory studies of the normal mouse liver have shown that terminal hepatic venules (ThV) and terminal portal venules (TPV) alternate at the margin of the liver (Fig 1a). The sinusoids connecting afferent to efferent vessels can clearly be seen. At higher magnification the nuclei of endothelial cells are visible along the sinusoids (Fig 1b). Individual red blood cells (rbc's), platelets, and white blood cells can be clearly distinguished.

b) Microcirculatory changes in the MHV-3 infected Balbc/J mouse.

The earliest sign of MHV-3 infection in-vivo is the appearance of localized areas of sinusoids with decreased red blood cell (rbc) velocity and the appearance of focal areas of edematous hepatocytes. The rbc's can be seen to move at normal velocities until within 2 or 3 sinusoids of the swollen hepatocytes. Flow then slows through the area of edematous hepatocytes and recovers on the further side (Fig 2a). These areas are seen as early as 6-8 hours post-infection. At some point flow ceases through the sinusoids in this area, and the red cells are rerouted around the area of blockage. A larger lesion is shown at low power in Fig 2b. In such cases, flow in adjacent sinusoids at the edge of lesions can be of greatly disparate velocities (as in Fig 1b, see part d below).

c) Carbon labelling of phagocytic cells.

Some mice were given India Ink (0.1 ml of 1/10 dilution) intravenously prior to in-vivo microcirculatory studies. Within a few minutes particles could be seen attached to the sinusoidal lining cells (Fig 3a). Since the particles were almost continuous, it appeared that all sinusoidal lining cells have the capacity to at least adsorb small particles. Within a few hours the particles were concentrated in areas within the cells. At 48 hours after
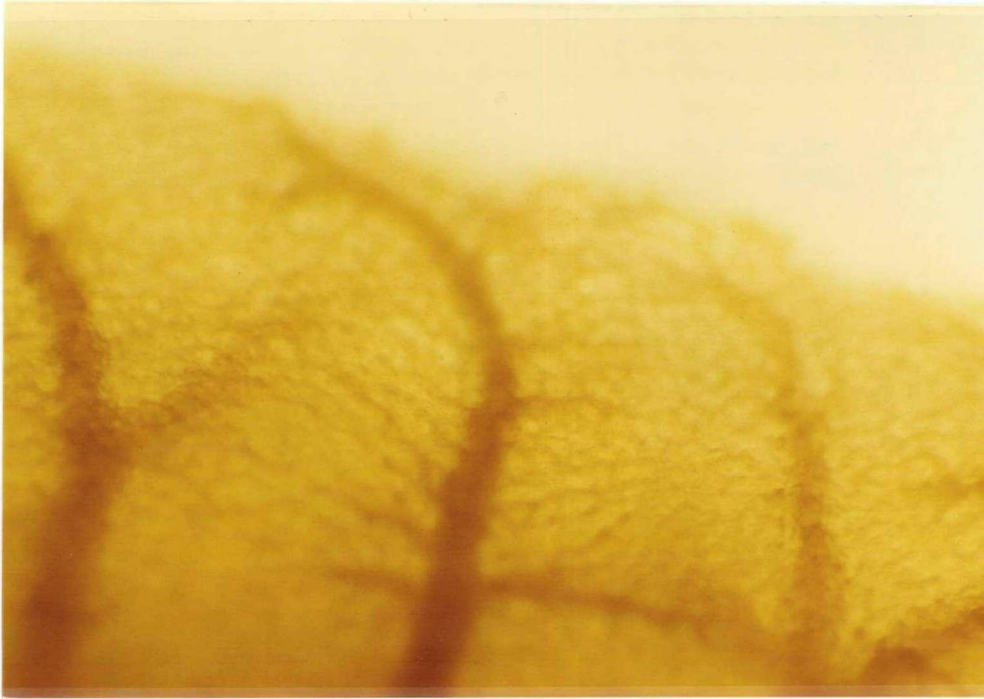
Figure 1a) In-vivo transillumination of a normal Balbc/J mouse liver , with alternating terminal portal and hepatic venules at the liver margin. (X 220).
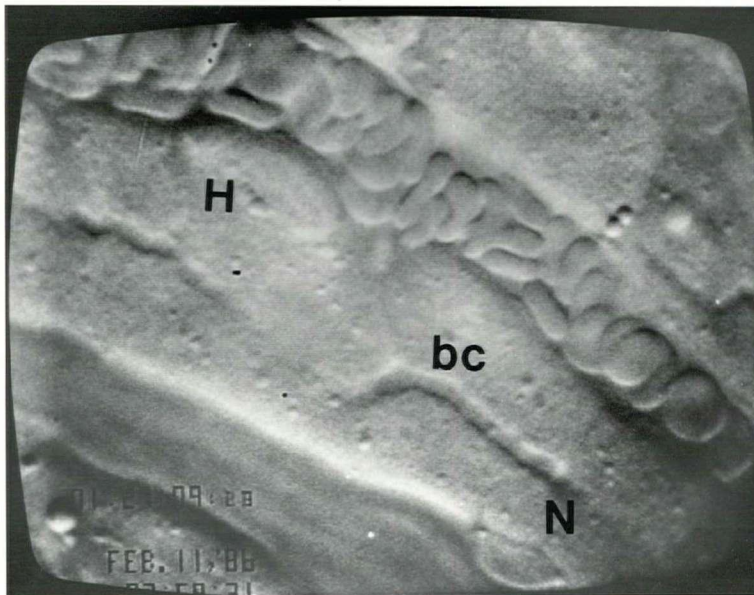
Figure 1 b) In vivo transillumination of normal Balbc/J mouse liver. Red cell flow is slower than normal in the upper sinusoid. H= hepatocyte, N=nucleus, bc=bile canaliculus (X2100).

Figure 2 a) In vivo transillumination of an MHV-3 infected Balbc/J mouse liver. Flow through edematous hepatocytes in the centre is slower than normal, sinusoids to the left and right have near normal flow (X 1320).
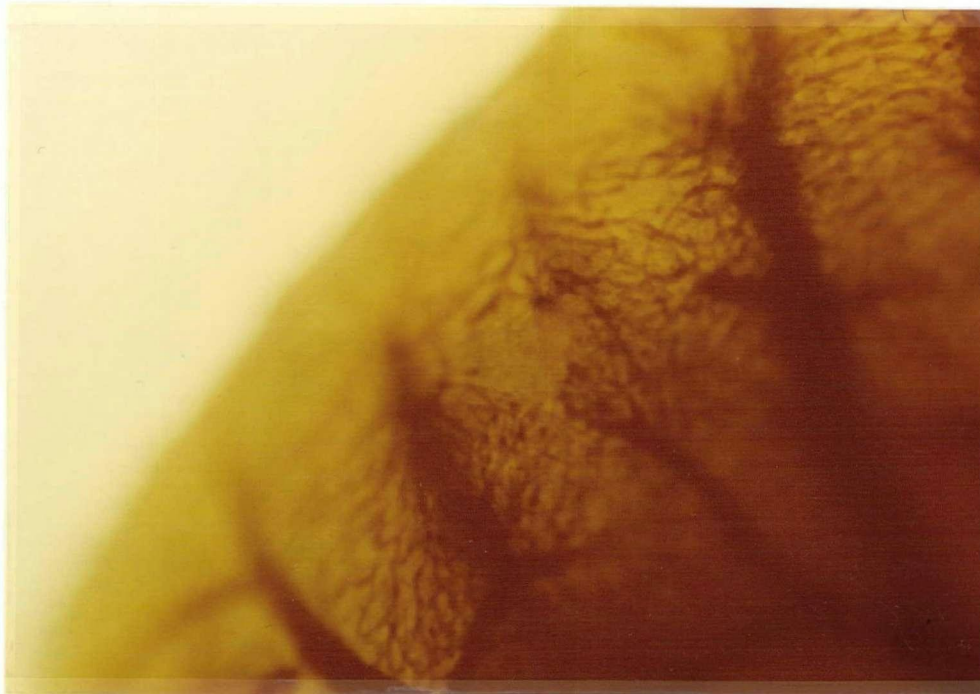
Figure 2 b) In vivo transillumination of the liver margin in an MHV-3 infected mouse, the pale area is without red cell flow (X 220).

Figure 3 a) In vivo transillumination of a Balbc/J mouse liver 10 minutes after intravenous carbon. Particles have adhered along the sinusoidal margins (X 2100).
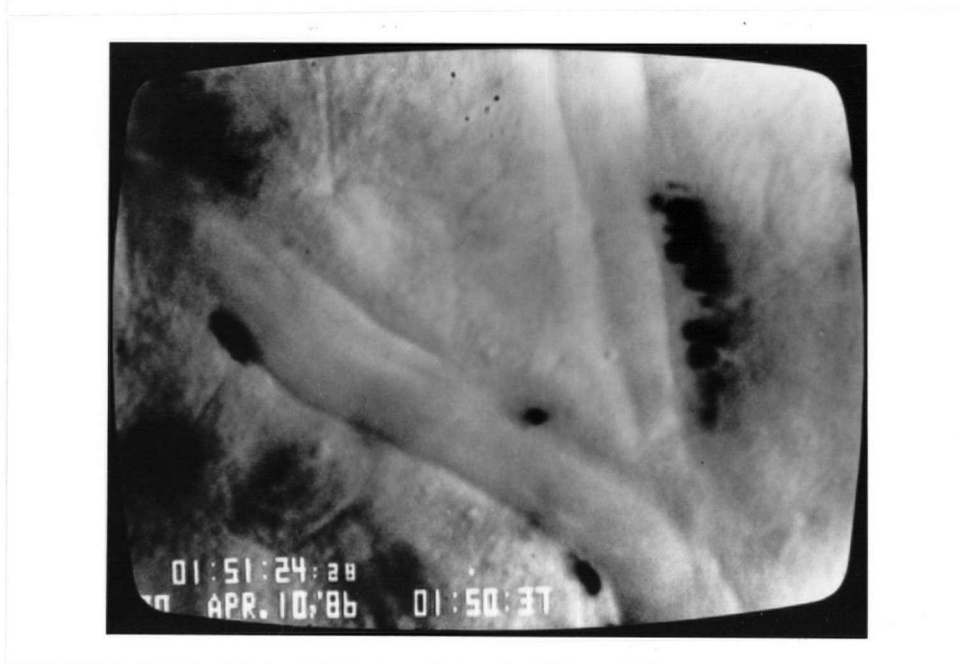
Figure 3 b) In vivo transillumination 48 hours after carbon injection. Carbon particles are now concentrated in the Kupffer cells (arrow) (X 2100).

28
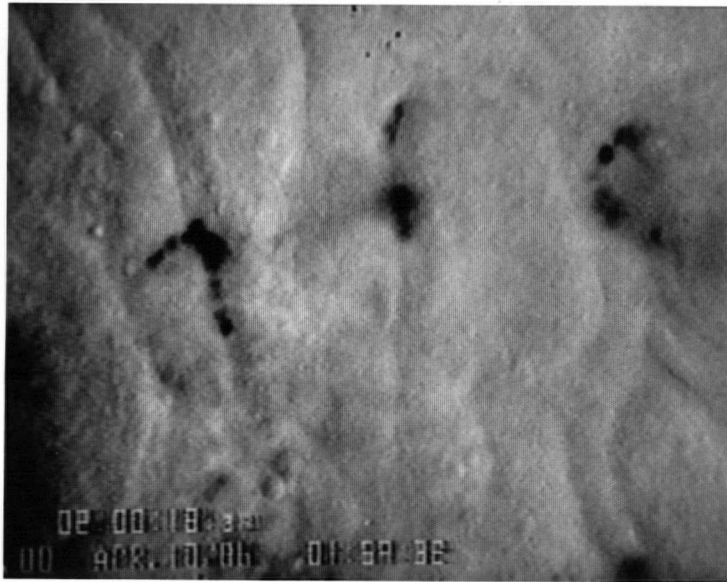
Figure 4 a) In vivo transillumination 48 hours post carbon. Particles are visible in Kupffer cells stretched across adjacent sinusoids, the central one is below focal plane (X 1320).
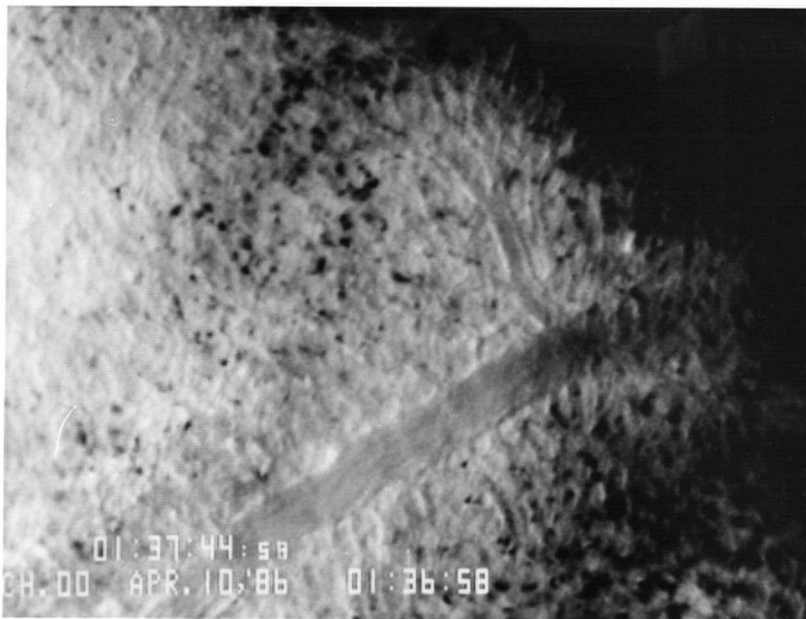
Figure 4 b) In vivo transillumination 48 hours post carbon. Kupffer cells are concentrated in Z3, the area closest to the hepatic venule in the centre (X 420).

injection, the particles were seen mainly in Kupffer cells, which now were visible as dark bands stretched along (Fig 3b) or across (Fig 4a) the sinusoids. Low power micrographs show that the Kupffer cells are much more densely distributed in Zone 1 of the acinus (Fig 4b).

d) Red blood cell velocity measurements.

Velocities of red blood cells were recorded in the sinusoids of both normal and infected mice from video recordings of flow taken at high magnifications . Flow in normal mice was measured in sinusoids from all zones of the acinus, and therefore represents a mean across the acinus. In infected mice, the flow was measured in consecutive sinusoids adjacent to and aligned with the periphery of a lesion, beginning with the most proximal sinusoid with visible flow (considered the 'first sinusoid') and moving outward from the lesion (as in Fig 1b, where the upper sinusoid has slow flow, and the lower sinusoid had normal streamlined flow). The values are recorded in Table 1. The mean velocities in the first and second sinusoids were significantly lower than the mean velocity in normal liver (Student's t test), whereas the mean velocity in the third sinusoid was not significantly different from normal values. This demonstrates that RBC velocity falls from normal to zero over a narrow border zone surrounding the lesion. The width of this zone coresponds to a maximum of three sinusoids (i.e. three to six hepatocytes).

e) Movement of cells against flow

During the course of the in-vivo microcirculatory studies it was observed that certain cells were able to move upstream against the pressure of blood flow. These cells were larger than red cells, and more granular in appearance . If the cells were observed over a period of minutes to hours, they sometimes were seen to move across a sinusoid and completely block the flow. The normal width of a mouse liver sinusoid is between 5-7 um. In addition, these cells could be seen to move upstream against flow. In the case shown in Fig 5 a-d, the cell moved upstream, and through a sinusoidal junction, temporarily blocking flow, before moving around a corner and down to the left.

TABLE 1

RBC Velocities in Sinusoids Adjacent to Lesions, and in Normal Livers

Measurement Site	Velocity (mm/sec ± SD) N=40-60
First (adjacent) sinusoid	17.4 ± 6.7 *
Second sinusoid	33.9 ± 8.7 *
Third Sinusoid	66.6 ± 27.3 †
Normal Liver	69.2 ± 30.6

These sinusoids were aligned with the periphery of individual lesions. RBC velocities were measured in consecutive sinusoids, beginning with the most proximal (i.e., first) sinusoid with visible flow and moving outward from the lesions.

* Significantly different from mean velocity in normal liver (P < 0.0005).

† Not significantly different from mean velocity in normal liver (P > 0.05).

The cell velocity was approximately 12 um/min. These cells are able to change shape quite rapidly; in Fig 5a this cell is globular and in b, 44 seconds later the cell is elongated, in another 11 seconds it has blocked the sinusoidal junction. These cells are considered to be Kupffer cells on the basis of their size , texture (from granular to distinctly ruffled), and ability to adhere for long periods of time to the vascular wall and to move along the cell surface without the rolling motion characteristic of neutrophils .

Liver histology and characteristics of the infiltrate

a) Normal Balb/cJ mice.

Histology of the normal Balbc/J mice has been previously reported in this model (Levy et al 1983) and for A/J mice by McNaughton and Patterson (1980). A section of normal liver can be seen in Figure 6a. The normal distribution of Mac 1+ cells can be seen in Fig 7a. A/J mice have fewer Mac 1+ cells (vide infra). The location of Mac 1+ cells parallels the distribution of Kupffer cells as seen by carbon-labelling, i.e. scattered, but denser in zone 1.

b) MHV-3 infected mouse liver

A detailed description of the pathology has been previously reported (McNaughton and Patterson 1980, Levy et al 1983). Briefly, light microscopy shows that by 12 hours after infection with MHV-3 there are focal areas of necrosis with a sparse mononuclear cell infiltrate. By 3 days p.i. they consist of large rounded areas of necrotic cells , with an occasional mononuclear cell at the periphery (Fig 6b). They are more commonly seen near portal vessels.

c) Immunofluorescent studies

Immunofluorecent studies showed the normal mononuclear cell population in the livers of non-infected Balbc/J mice to consist almost entirely of Mac-1 positive cells (i.e. of the monocyte/macrophage lineage) (Fig 7a) . The normal number of Mac 1+ cells (2.5 + 1.0 SD) per high power field (HPF) increased by greater than twofold by 12 hours p.i.

Figure 5 In vivo transillumination of a Balbc/J mouse liver. a) A Kupffer cell (arrow) has become elongated and is moving b) downward through the sinusoid. In c) the sinusoidal junction is completely blocked. A second Kupffer cell can be seen below it (short arrow). d) The first Kupffer cell has moved through the junction and downwards, and normal flow has resumed (X 1100).
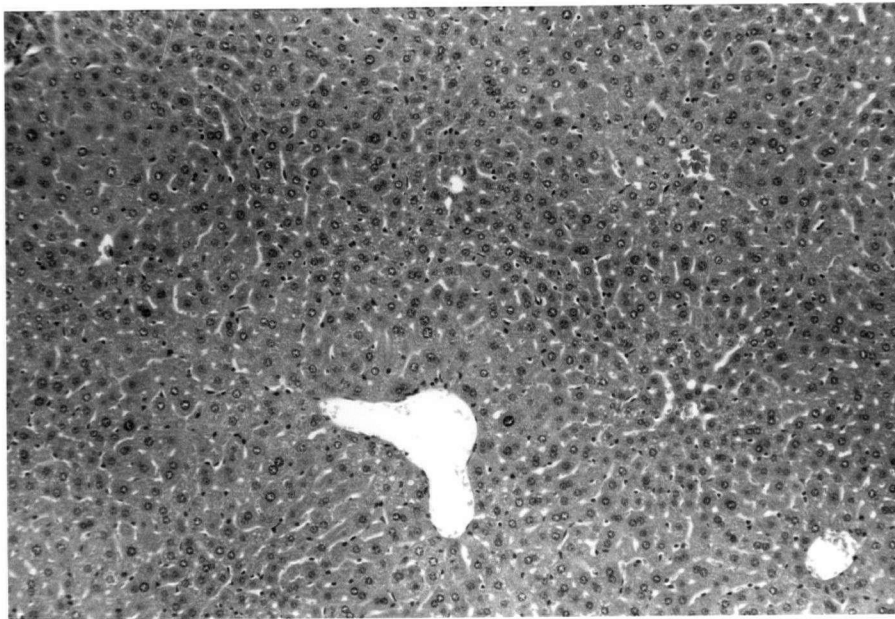
Figure 6 a) Normal liver histology in the Balbc/J mouse (x 210).
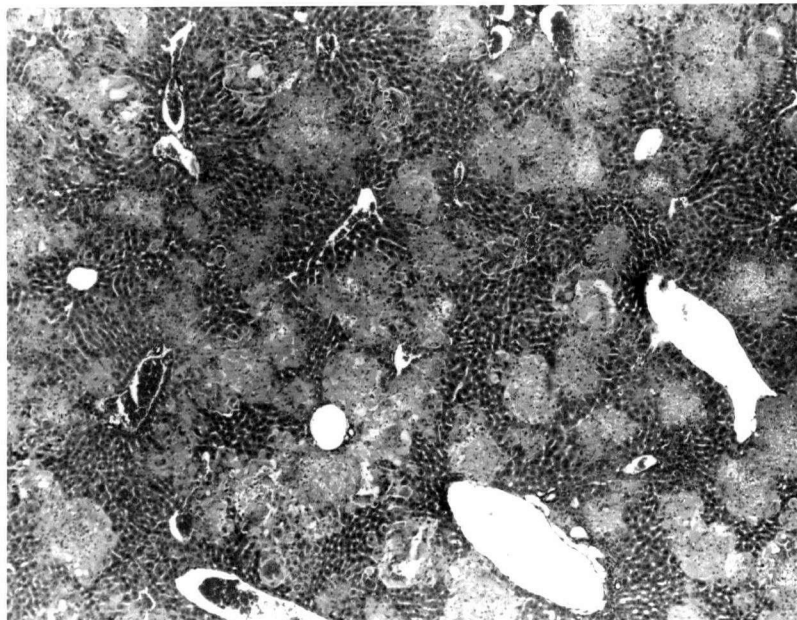
Figure 6 b) Liver of a Balbc/J mouse 3 days post-infection with MHV-3. Cross sections of the areas of necrosis are rounded and beginning to coalesce (X 210).

(fig 7b) (p<.025), and were more than twentyfold greater (>50 cells per HPF) at 3 days post-infection (p<.001) (Fig 8a).

In the resistant A/J control mice the normal number of Mac 1+ cells was lower than in uninfected Balbc/J (1.3 + 1.0) vs 2.5 ± 1.0 (p< 0.25). However, in contrast to Balb/cJ animals, the number did not increase up to 4 days post-infection (2.6 ± 2.7 n=10 mice, P=NS).

Lymphocytes expressing Thy-1, Lyt-2 or L3T4 antigens were uncommon in the hepatic parenchyma of both normal or infected A/J or Balb/cJ mice at any time point post-infection and, when present, were usually located in an hepatic sinusoid or larger vessel (Fig 8b).

We were unsuccessful in our attempts to locate Ia postive cells using the MAS 053C antibody in these frozen sections, although it did stain the expected number of positive cells in a viable spleen cell suspension from the same mice. Whether this was due to loss or modification of the antigen in the frozen sections has not yet been established.

Transmission Electron Microscopy

Transmission electron micrographs taken at 12 hr post infection show that some hepatocytes exhibited an unusual number of vesicles containing whorled membranes (Figure 9a). Some sinusoids appear to be blocked with cells (Figure 9b). Ito, or fat storing cells, are frequently seen between adjacent hepatocytes (Figure 9a,b). Although some hepatocytes retain their normal polygonal shape, others show varying degrees of ballooning degeneration characterized by convex contours (indicating cell swelling), enlargement, and lower overall electron density (Figure 10a). These hepatocytes have somewhat dilated smooth and rough endoplasmic reticulum, swollen

Figure 7 a) Normal population of Mac 1+ positive cells in a Balbc/J mouse liver (X 210).
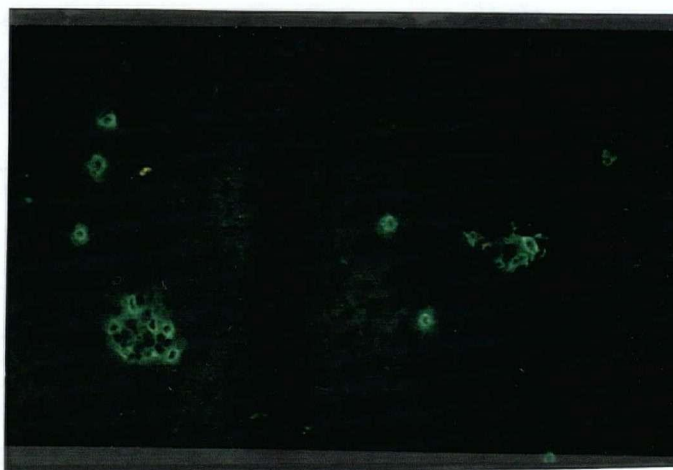
Figure 7 b) Mac 1+ cells in the liver of the Balbc/J mouse 12 hours post infection with MHV-3 (X 210).
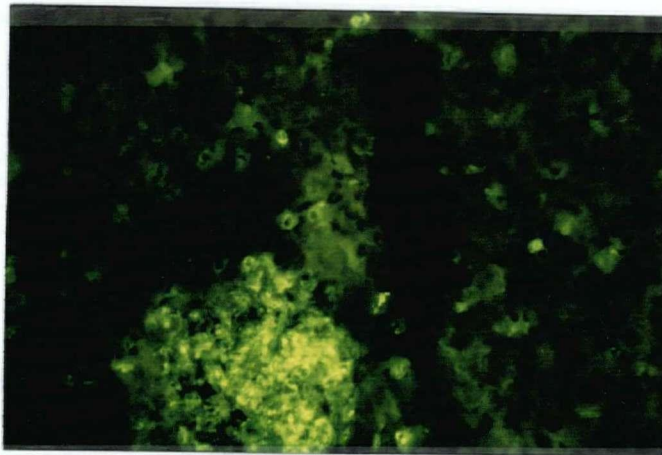
Figure 8 a) Mac 1+ cells in the liver of a Balbc/J mouse 3 days post infection with MHV-3. Antibody is also adhering to the foci of necrosis (X 420).

Figure 8 b) L3T4 postive cells in the MHV-3 infected Balbc/J mouse liver 24 hours post-infection. The only positive cell was seen in a venule (X 210).

mitochondria with increased intercristal distance, and loss of matrix granules. Some cells possess dense aggregates in the mitochondria. In some cases the outer mitochondrial membrane was no longer intact or associated with membranous whorls (10b). Bile canaliculi were often dilated and contained electron dense material (bile), and canalicular microvilli which were blunted, or decreased in number (Figure 11a,b). Ballooned cells showed a relative decrease in glycogen and an increase in cytolysosomes or areas of focal cytoplasmic degradation (Figure 12a).

From 24 hours to 3 days p.i., similar changes were seen. In fact, with the exception of atrophic degenerating cells (Figures 11b), and the appearance of acidophilic bodies (Figure 14b), most of the changes were clearly apparent from 12 hours on. By 36 to 48 hours, occasional swollen degenerating cells were seen, in this case apparently blocking a sinusoid (Figure 13a). Necrotic cell debris was seen both within lysosomes in the cytoplasm and taken up by Kupffer cells (Figure 13b). Some hepatocytes had lost the integrity of their cell membranes (Figure 14a). At this time, numerous peroxisomes with pseudocrystalline figures were common in the ballooning hepatocytes (Figure 14b).

Kupffer cells also exhibited dilated rough endoplasmic reticulum, peripheral condensation of the nucleus, and dilatation of mitochondria. The changes were similar to those seen in hepatocytes (Fig 9a), but more advanced , i.e. with definite rarefaction and floculant densities in the mitochondria . Nuclear changes occurred later than those in the Kupffer cell cytoplasm, and were advanced at 36 hrs post infection (Figure 13b).

Scanning Electron Microscopy

The surface of corrosion casts from liver of normal mice showed complete filling of sinusoids in a normal acinar arrangement (Fig 15a). In casts from infected mice, lesions appeared to be approximately spherical cavities, indicating where the casting compound
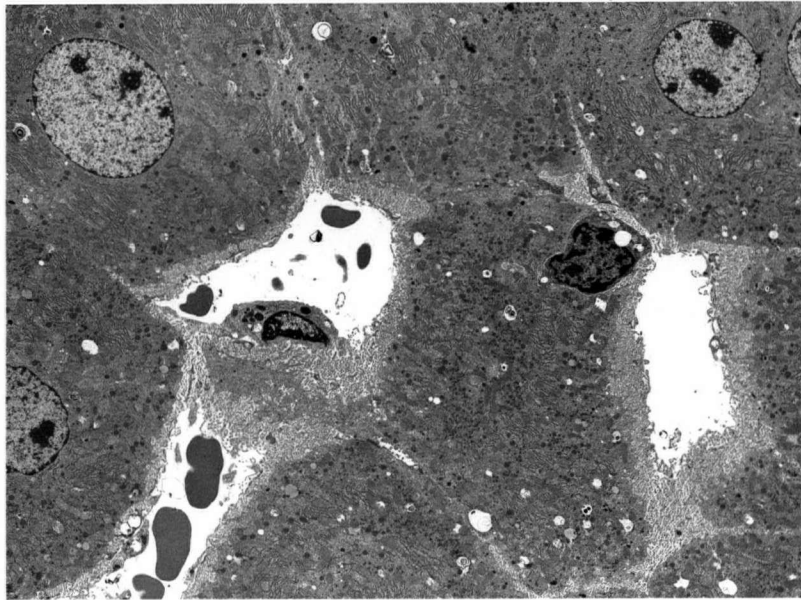
Figure 9 a) Transmission electron micrographs (TEM) of liver of MHV-3 infected mice at 12 hours post-infection. Hepatocytes with numerous vesicles, which contain whorled lamellar material (1,100 x).
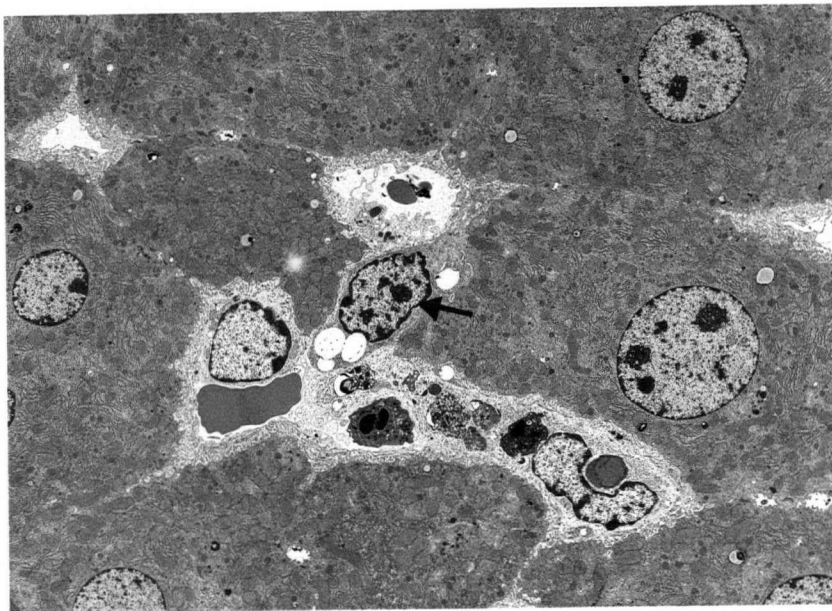
Figure 9 b) In localized areas, some sinusoids appear to be blocked with cells. A fat storing cell (FSC) is visible (arrow) (1,100 x).
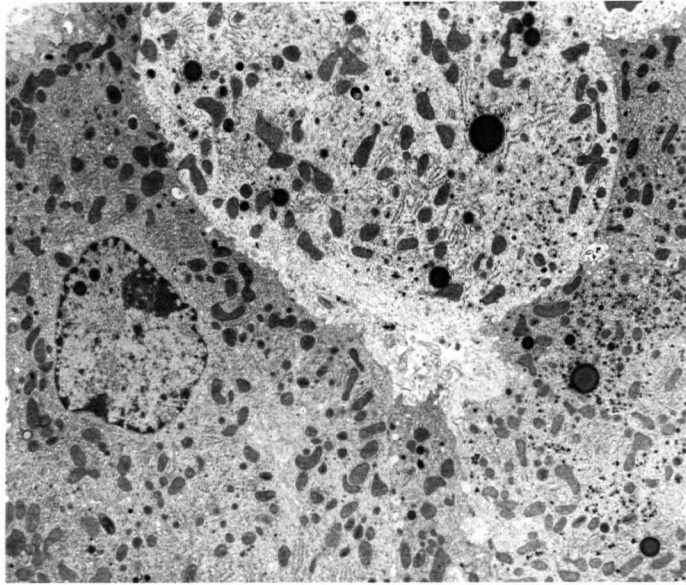
Figure 10 a) TEM 12 hours post-infection (p.i.) with MHV-3. Occasional hepatocytes are swollen as indicated by pale , vaculolated cytoplasm and convex contours. (3,000x).
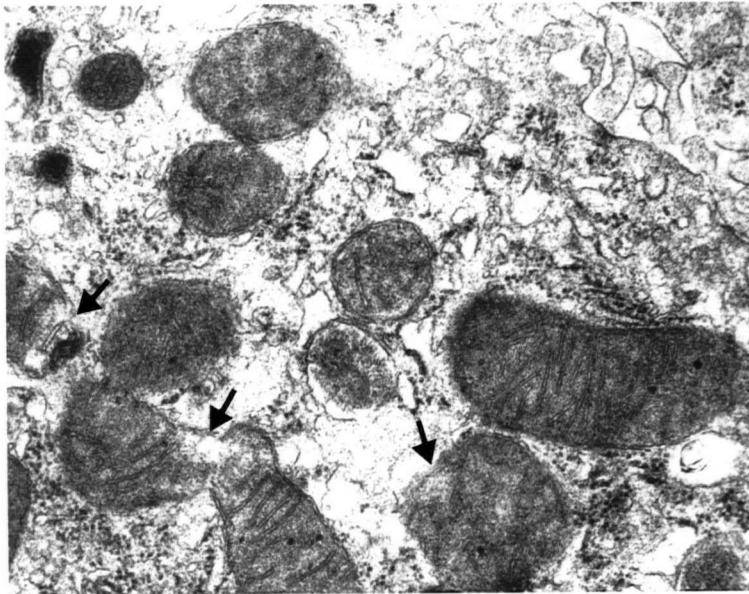
Figure 10 b) TEM of mouse liver at 24 hours p.i. with MHV-3. Areas where the mitochondrial membrane is disrupted or associated with membraneous whorls are indicated (arrows) (30,000x).
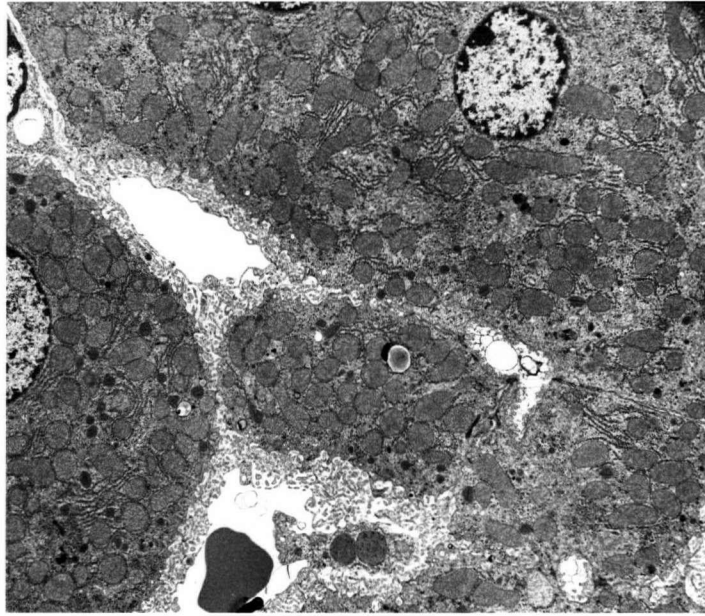
Figure 11 a) TEM of MHV-3 infected mouse liver at 24 hours p.i. with MHV-3. Bile canaliculi between the hepatocytes (HC) are dilated with blunted microvilli and electron dense deposits. One HC (centre) appears to be only loosely connected to its neighbouring hepatocytes (3,000x).
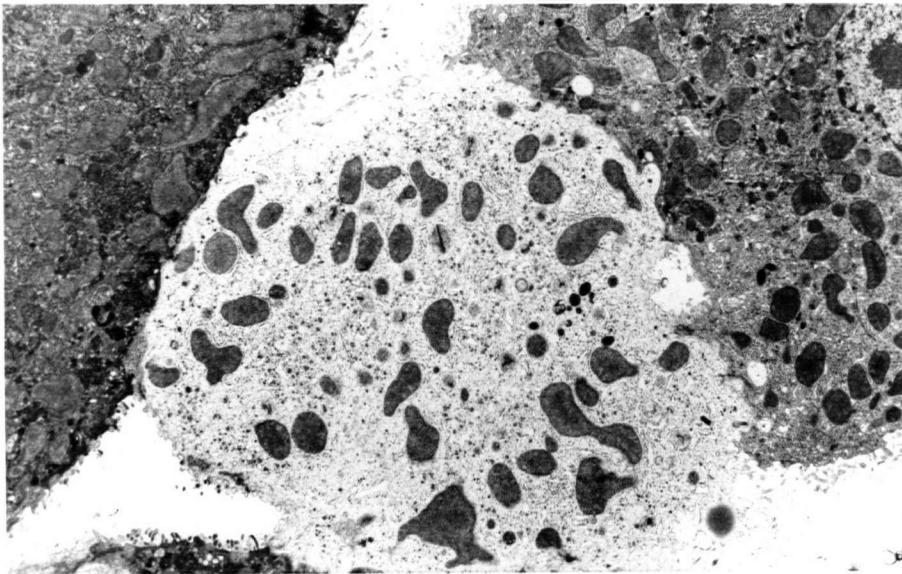
Figure 11 b) TEM of MHV-3 infected mouse liver at 36 hours p.i. A pale, swollen, vaculolated hepatocyte is apparently blocking a sinusoid (s). bc= bile canaliculus. (3,000x).
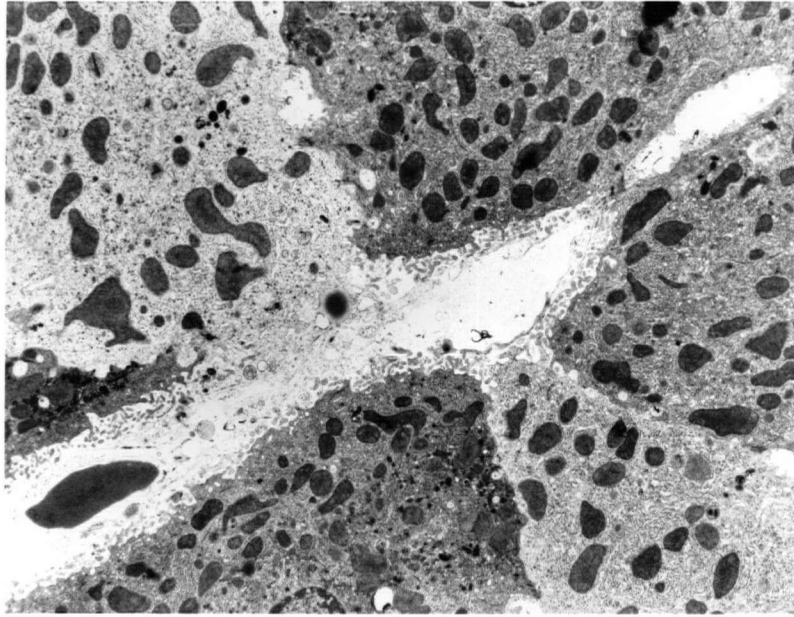
Figure 12 a) TEM of MHV-3 infected mouse liver at 36 hours p.i. An hepatocyte (ballooning degeneration) is swollen and partially obstructing a sinusoid. The cell membrane is no longer intact on the sinusoidal side (3,000x).
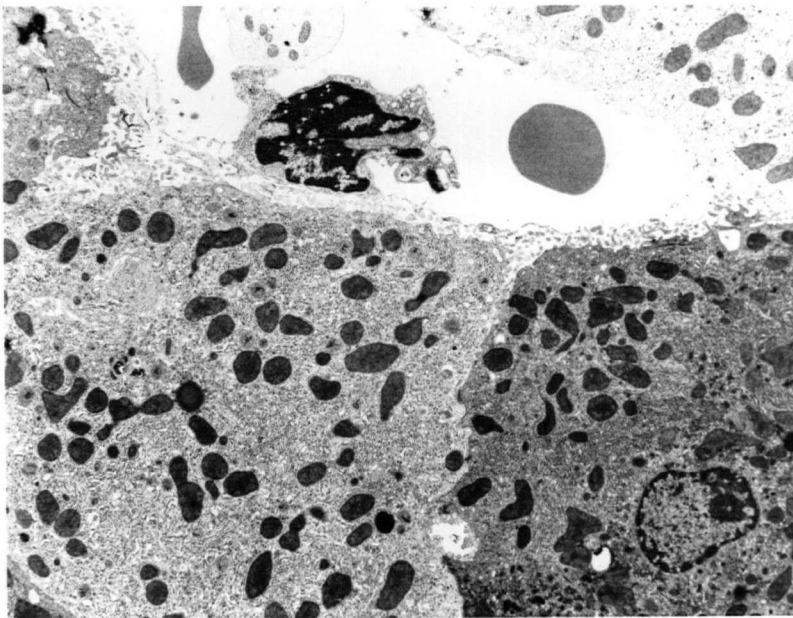
Figure 12 b) TEM of MHV-3 infected mouse liver at 36 hours p.i..Degenerating hepatocytes contain numerous lysosomes and peroxisomes (many with crystalline figures). An overlying Kupffer cell shows signs of nuclear degeneration, and condensation of chromatin (3,000x).
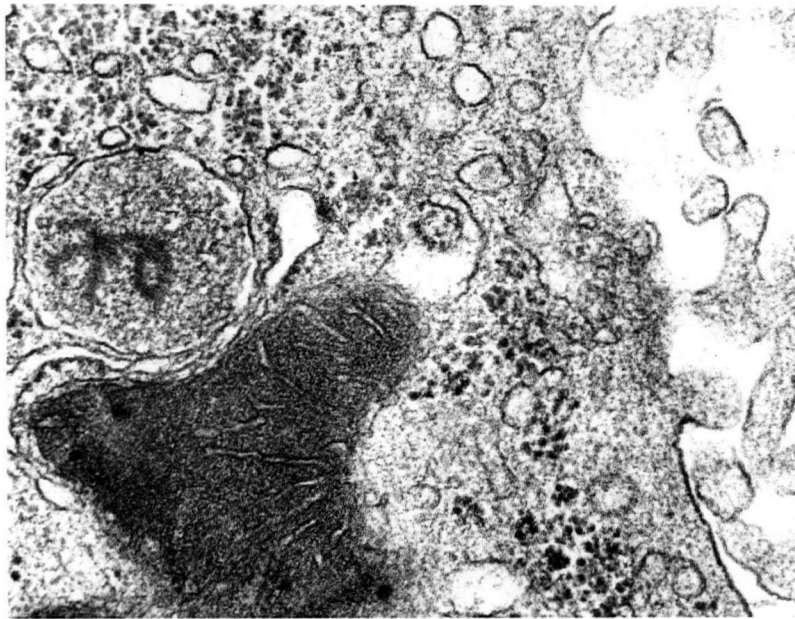
Figure 13 a) TEM of MHV-3 mouse liver 36 hours p.i. Higher magnification of peroxisome with a crystalline figure (50,000x).
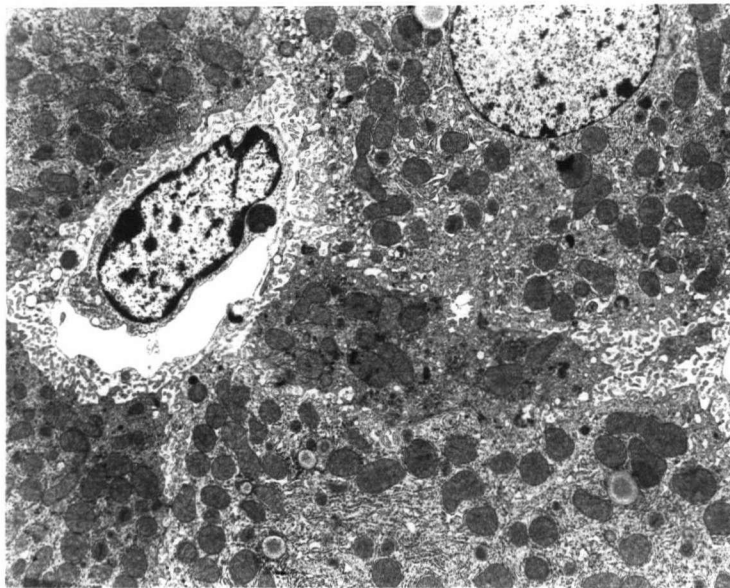
Figure 13 b) TEM of MHV-3 infected mouse liver 72 hours p.i. A section of an atrophic hepatocyte (HC) with slightly condensed cytoplasm is in the centre of the micrograph. A dilated bile canaliculus (arrow) is visible at the periphery of the HC. Dense aggregates are associated with the mitochondria in the atrophic HC (3,000x).
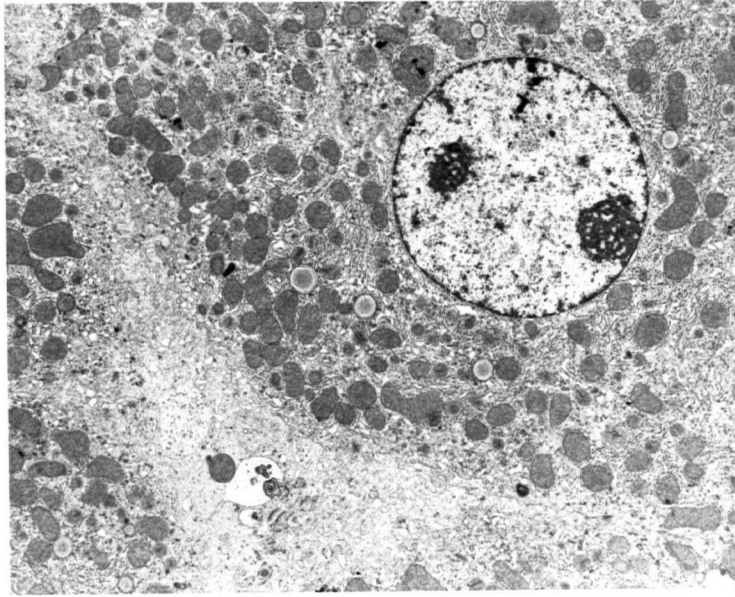
Figure 14 a) TEM of MHV-3 infected mouse liver at 72 hours p.i.. The space between two hepatocytes is filled with swollen blebbed microvilli, and some cell debris (3,000x).
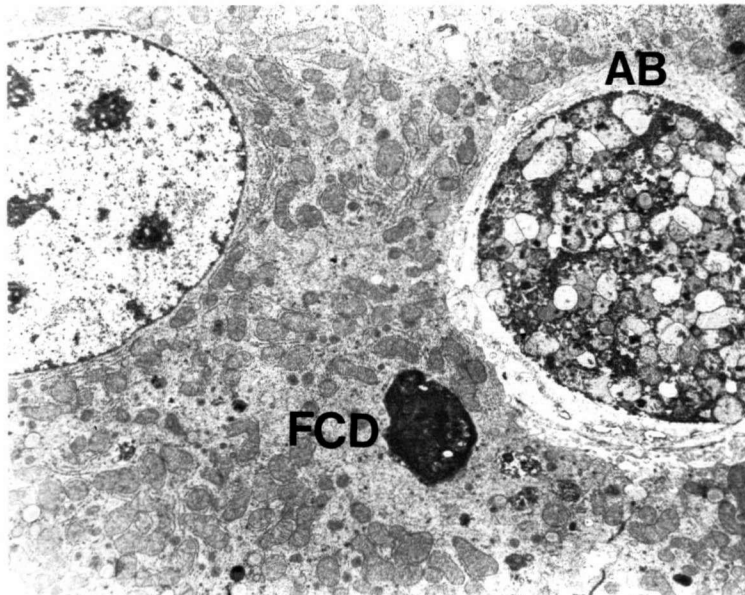
Figure 14 b) TEM of MHV-3 mouse liver 72 hours p.i.. An atrophic cell has become detached and formed a highly condensed acidophilic body (AB). Note an area of focal cytoplasmic degeneration (FCD) in a nearby HC (7,000x).

was unable to fill the sinusoids (Fig 15b). The size and distribution of the lesions was

determined by measuring these discrete areas from photomicrographs of the casts. At 48

hours post-infection the mean diameter was 83 ± 26 um (n=110), with a range of 44-178

um. The number of lesions/mm^2 corresponds to about 1 lesion per liver acinus (the area

supplied by one terminal portal venule). Views of microcorrosion casts of individual

lesions, obtained at higher magnification (Fig 16a), show many "blind ended" sinusoids

forming a distinct boundary between the perfused and non-perfused areas. At still higher

magnification many of these sinusoids were seen to have concave impressions at their

ends (Fig 16b) as if further passage of the material had been prevented by cells which

blocked the lumen. The size of these indentations (5-7 um) is consistent with that of red

blood cells.

In Vitro Lymphocyte Proliferation

In order to determine whether the functional differences between A/J and Balb/cJ mice

were related to the presentation and response to the virus by lymphocytes or

macrophages in these strains, unelicited peritoneal macrophages obtained from both

resistant A/J and susceptible Balbc/J mice at 6-8 weeks of age were infected with MHV-3

and tested for their ability to stimulate lymphocyte proliferation in vitro (Fig 17). The

difference between uninfected and MHV-3 infected groups was significant (p <.0005) for

all groups. The maximum response was obtained at 8 weeks of age in Balbc/J mice, and

thereafter declined. The stimulation index (S.I.) for Balbc/J splenocytes when presented

with autologous MHV-3 infected macrophages was 4.4. The response of the A/J mice

was about 4 times greater (S.I. = 20.5). To determine whether the lesser response in the

Balb/cJ cells was due to less effective antigen presentation by the macrophages or to an

impaired lymphocyte reponse to the virus, Balbc/J macrophages were tested against A/J

lymphocytes and vice versa. A/J macrophages plus Balbc/J splenocytes gave a similiar

response to A/J plus A/J, whereas Balbc/J macrophages plus A/J splenocytes gave a low
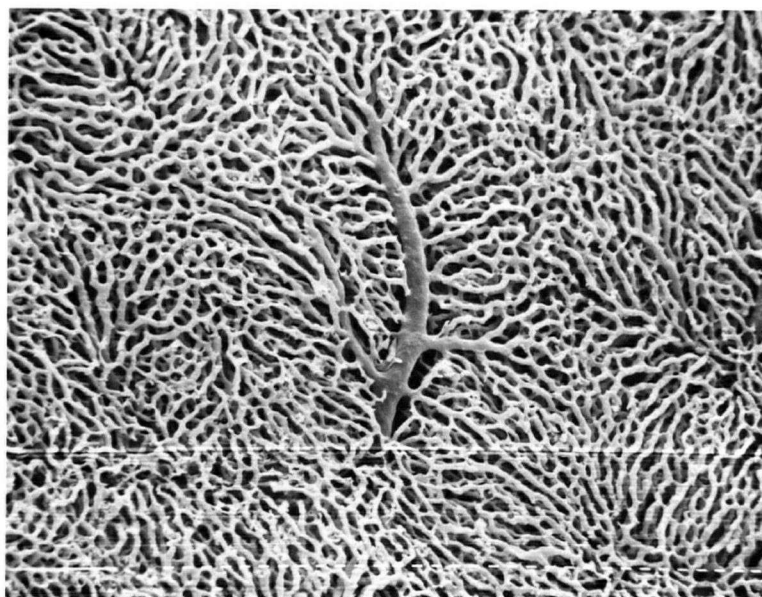
Figure 15 a) Scanning electron microscopy of the normal Balbc/J mouse liver (short bars = 10 um).
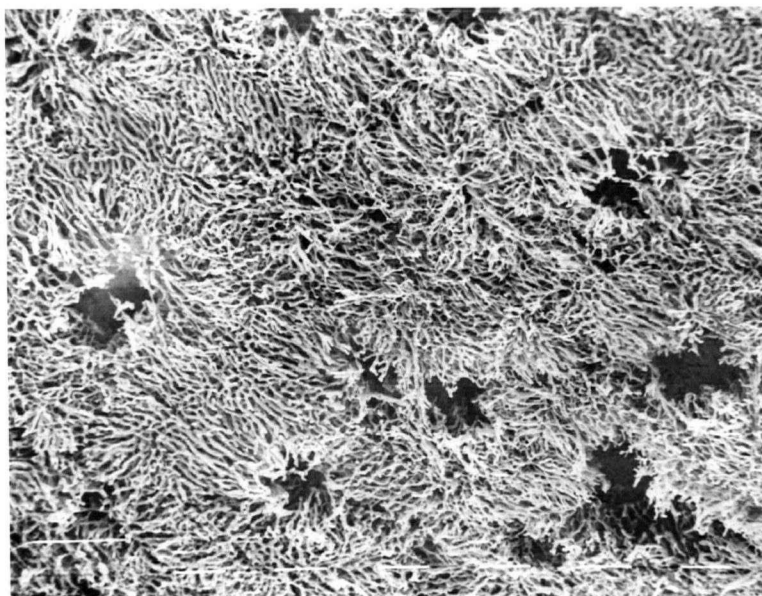
Figure 15 b) Scanning electron micrograph of a Balbc/J mouse liver 48 hours post-infection with MHV-3. Numerous areas were impermeable to the casting compound (bars = 100 um).
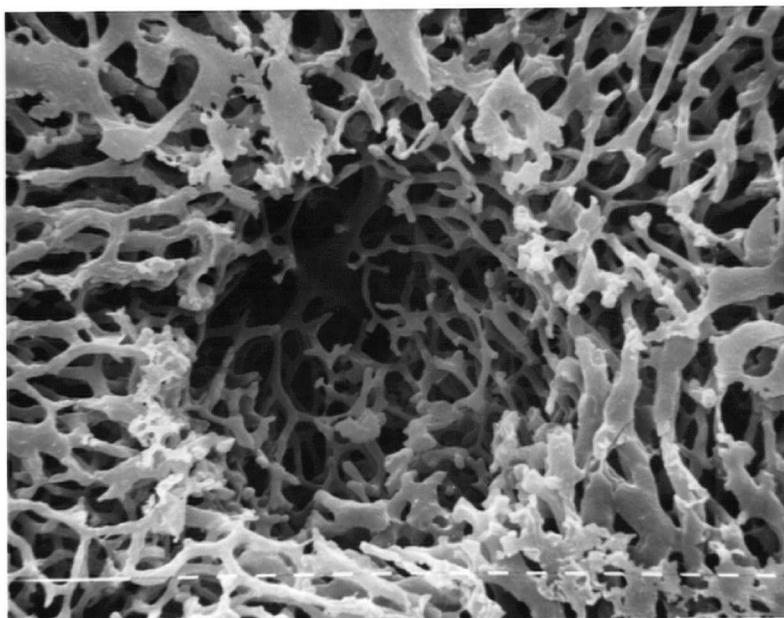
Figure 16 a) Higher magnification scanning electron micrograph of a single spherical lesion 48 hours post-infection with MHV-3 (bar = 10 um).
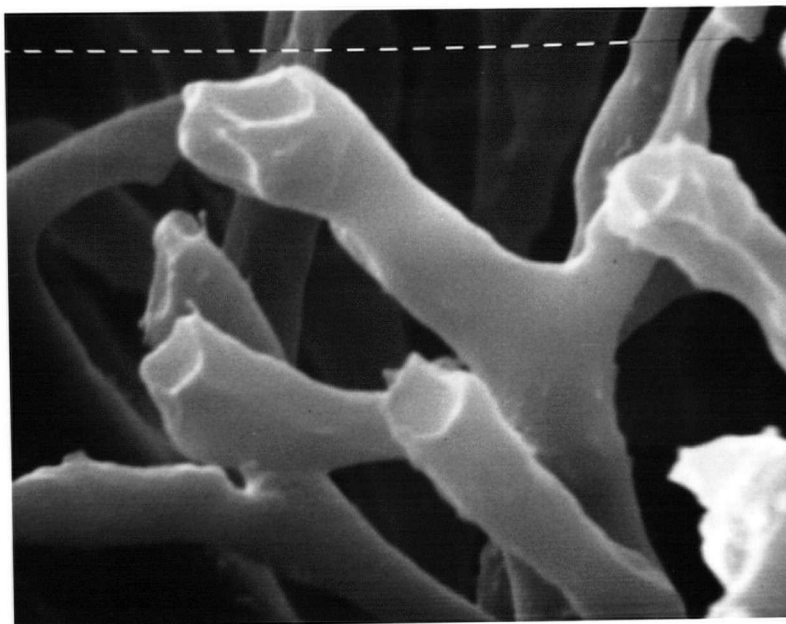
Figure 16 b) At very high magnification the ends of the sinusoids can be seen to have concave depressions, the size 5-7 um) and shape suggests abuttment of the casting compound against red blood cells (bar= 1 um).

response (S.I.= 6.1x). The maximum of response of 30,000-40,000 cpm over a 48 hour incubation period was highly significant (p<.0005 and an S.I. of over 100), compared to the uninfected control.

Cyclosporine A Treatment

Although the histological studies indicated that no T-cells were present in the lesions, in order to eliminate a potential systemic role for T-cells, cyclosporine was administered. Cyclosporine (CsA) treated mice became clinically ill (apathy, ruffled fur) earlier than the MHV-3 only control. Mean survival time in the mice receiving CsA + MHV-3 was 4.8 $\pm$ 0.5 (mean $\pm$ SEM) days, while in those receiving MHV-3 only it was 5.3 $\pm$ 1.4 days (P=NS) . The histological picture was similar in both groups, with large areas of hepatocellular necrosis with little cellular infiltrate. The slight (but not statistically significant) difference in the amount of necrosis recorded (Table 2) may reflect the fact the CsA-treated mice required slightly earlier euthanasia. A second possibility is that cyclosporine may have a dampening effect on release of free radicals (Kahan 1989).

Detection of Anti-MHV Antibodies

a) Immunofluorescence

Immunoflorescence was employed to investigate the possibility that immunoglogulin or immune complexes representing a humoral response to the virus might be present in the livers of Balbc/J mice after MHV-3 infection. Surprisingly, granular deposits were evident along the margins of the sinusoids of both normal uninfected 6 week old Balbc/J mice (Fig 18a), and those recently infected (12-24 hours previously) (Fig 18b). Sometimes the deposits in the infected mice appeared to be of greater intensity, although there was no strong correlation between intensity and time post-infection. In addition, the resistant A/J mice also showed a similiar pattern of immunofluorescence both pre- and post-infection (Fig 19 a,b).

TABLE 2

Effect of Cyclosporine A on
MHV-3 Infection

Treatment	Mean Survival Time (days)	% Necrosis	CsA (ng/ml)
MHV-3 only	5.3 ± 1.6	55 ± 29	—
MHV-3 + CsA	4.8 ± 0.6	21 ± 12	3697 ± 1162

There were 5 Balbc/J mice per group.

CsA = Cyclosporine A. 70 mg/kg/day in olive oil given orally. Serum levels were measured during trough 24 hours post administration.

P = N.S.
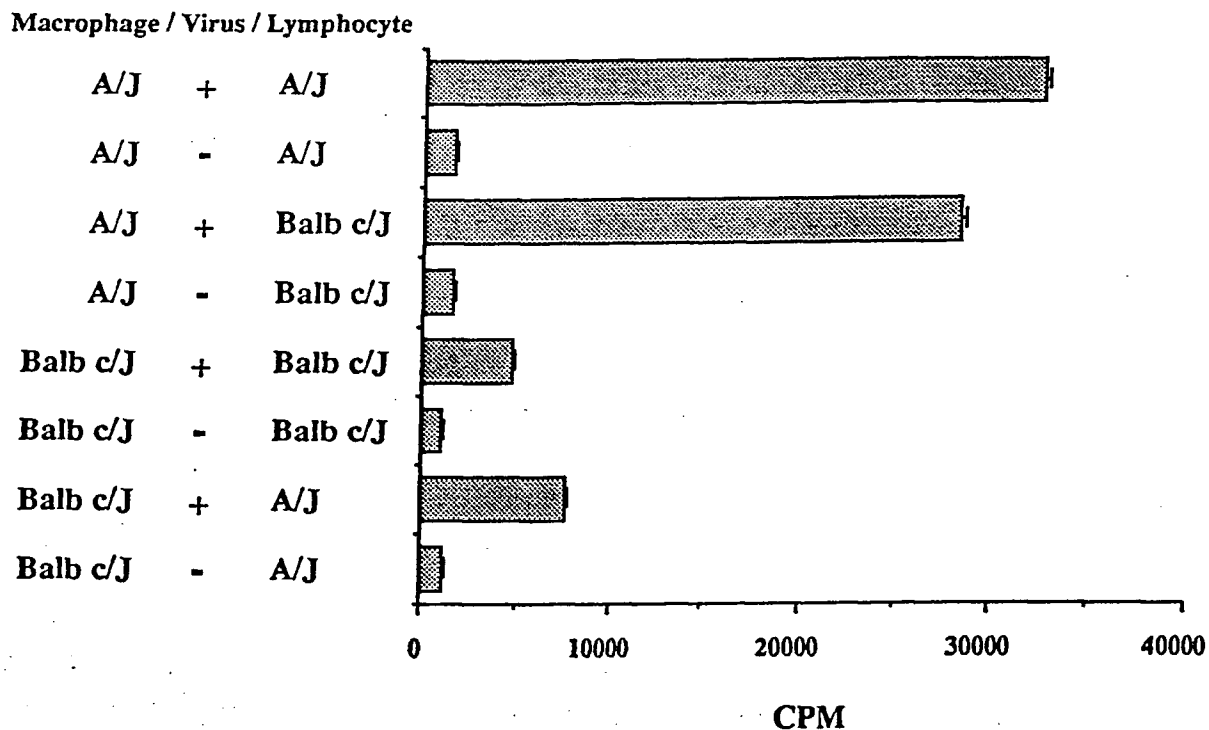
Fig 17 Ability of normal or infected Balbc/J and A/J macrophages to stimulate lymphocyte proliferation in vitro. Peritoneal macrophages (10^5) were exposed in vitro to MHV-3 or medium for 30 min, then excess virus was removed and 2×10^6 splenic lymphocytes were added and incubated for 48 hours, followed by 18 hours with ^{3}H thymidine.
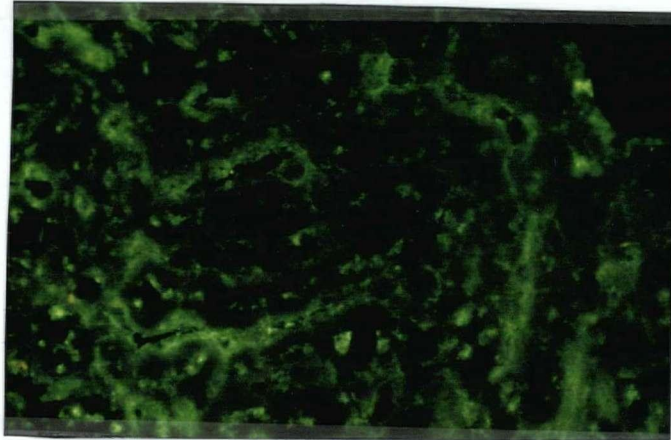
Figure 18 a) Immunofluoresent antibodies to IgG show faint granular deposits along the sinusoids of normal Balbc/J mice (X 420).
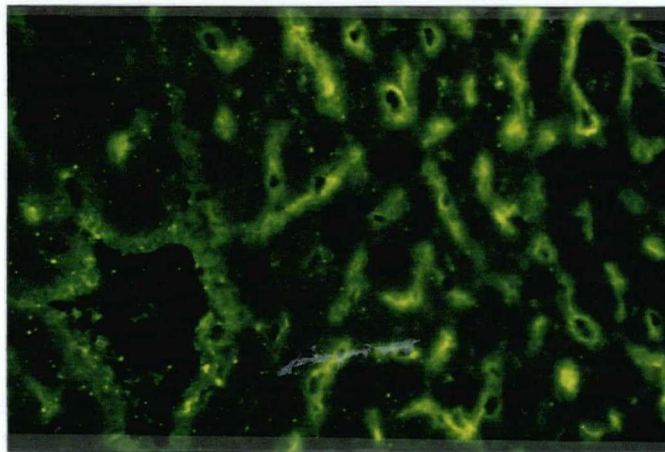
Figure 18 b) Intense but scattered deposits of IgG are seen in the sinusoids of Balbc/J mice 3 days post infection with MHV-3 (X 420).
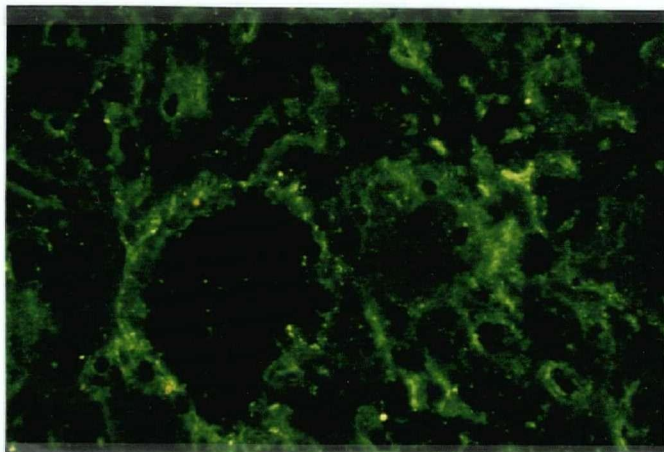
Figure 19 a) Normal A/J mice also show coarsely granular IgG deposits in the sinusoids of uninfected mice (X 420).
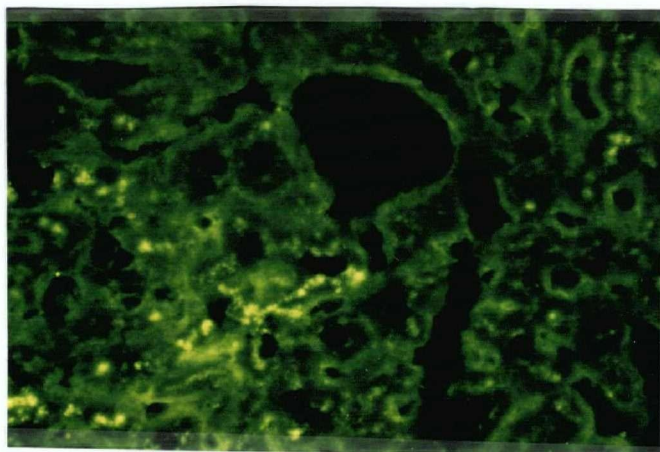
Figure 19 b) Intense IgG deposits in A/J mice 3 days post-infection with MHV-3 (X 420).
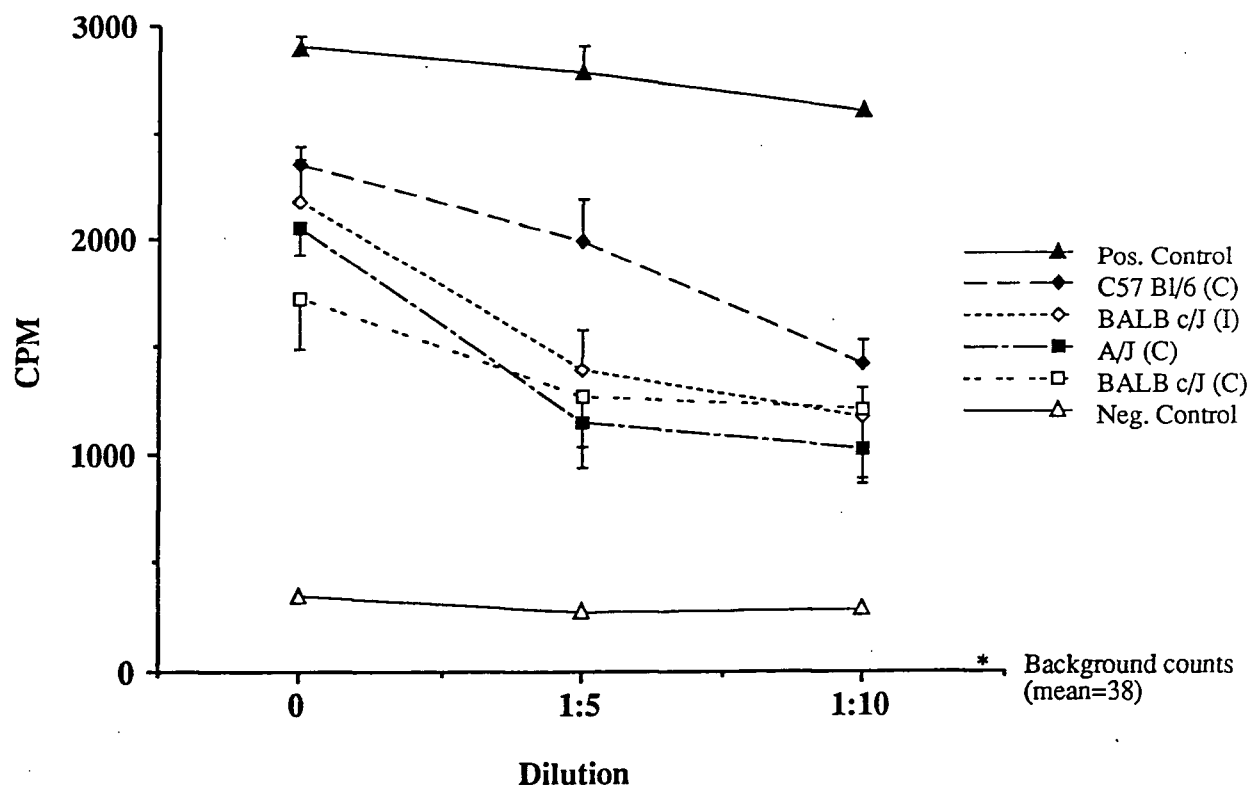
Figure 20 RIA for antibody to MHV. The positive control was polyclonal rabbit anti mouse antibody to JHMV (MHV-4), and the negative control was uninfected L-cell lysate. Normal mice of three strains plus Balbc/J mice infected with MHV-3 were all positive for antibody to MHV.

b) RIA

Serum from both normal and MHV-3 infected mice (A/J, Balbc/J, or C3HebFe/J) was tested to confirm the presence of antibody to MHV. All three strains of mice were found to possess antibody cross reactive with MHV-3 both prior to and following infection (Fig 20). Since completely MHV free mice were unavailable , it was impossible to have a totally negative control. Only if such mice were available would it be possible to determine the effect of a primary infection with MHV-3.

Chemiluminescence

The response of uninfected A/J and Balb/cJ mouse peritoneal macrophages to stimulation with opsonized zymosan was the same (Table 3), i.e. a slight increase that peaked at 40 to 50 minutes post-infection respectively . The infected Balbc/J mice had a somewhat increased basal level of chemiluminescence in their resting macrophages pre-zymosan stimulation. Chemiluminescence is the decay of excited oxygen species to ground state, with the emmission of energy as light which can be captured directly and measured, or amplified by the light emmittor luminol. The increased basal level may be a result of the macrophages already having been activated in-vivo by a prior infection with a non-virulent MHV strain, such as MHV-S. The MHV-3 infected Balbc/J macrophages responded strongly to zymosan stimulation far exceeding that of the uninfected cells or of MHV-3 infected A/J macrophages (p<.0005). The peak time for chemiluminescence was similar to that seen in uninfected groups, i.e. 55 minutes. A representative experiment is shown in Fig 21. Although the absolute count of the peak response differed from batch to batch of cells, the pattern was similar, and Balbc/J infected cells always responded with a highly significant increase in chemiluminesce.

Oxyradical Reaction Products

To establish free radical mediated injury, livers were examined post-infection for the

TABLE 3

Chemiluminescence Pre- and Post-Infection
with MHV-3

	Balbc/J Control	Balbc/J Infected	A/J Control	A/J Infected
Number of mice	4	4	5	5
CPM pre-zymosan	20 ± 5	308 ± 84	34 ± 9	30 ± 12
Peak CPM post-zymosan	98 ± 14	37,478 ± 4,210	58 ± 19	92 ± 6
Fold increase over control	5	122	1.7	3
Time at peak	50 min	55 min	40 min	50 min

Each experiment was performed in triplicate

CPM = mean ± SEM
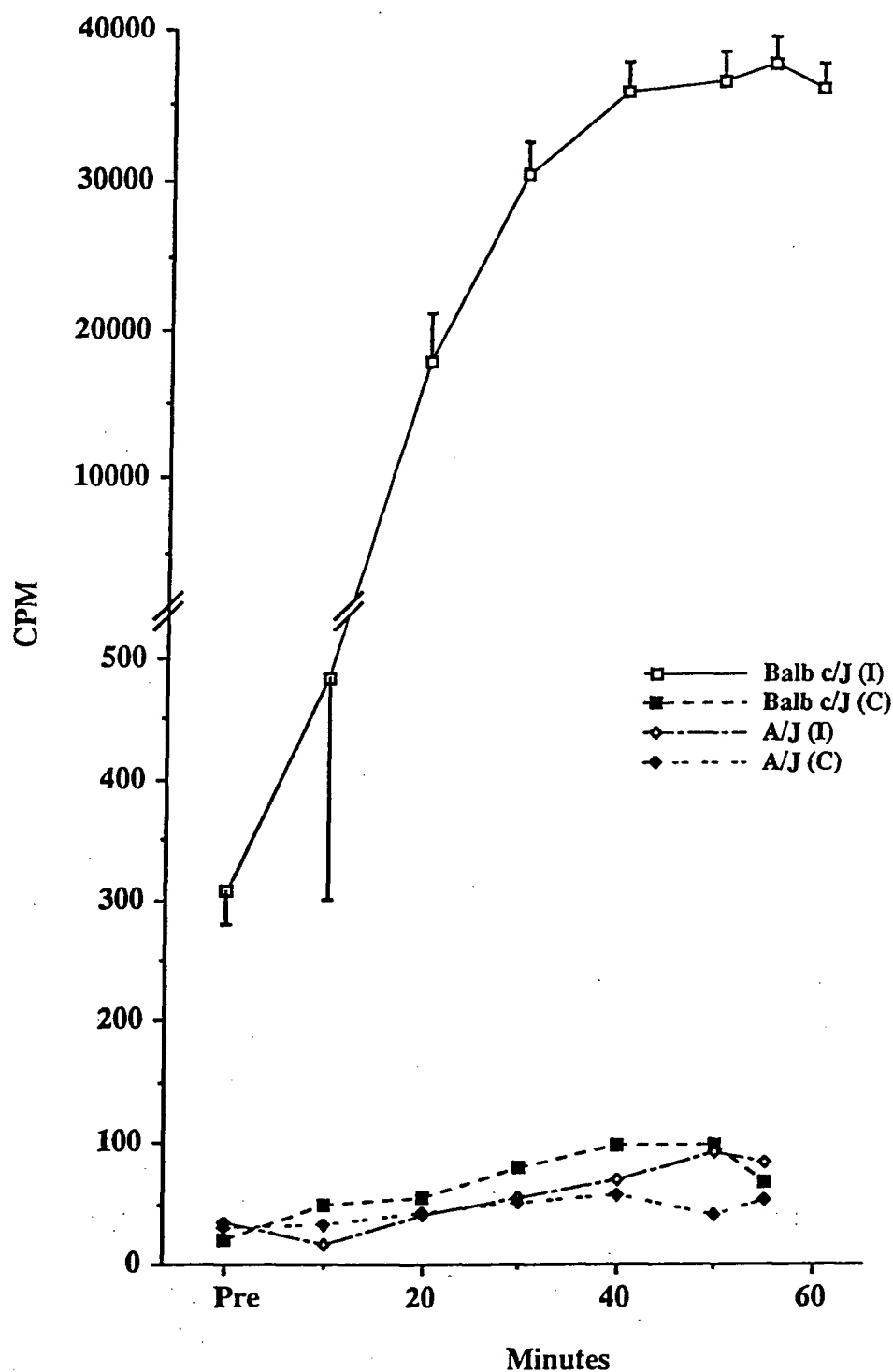
Figure 21   Time course of chemiluminesence of in-vivo uninfected and MHV-3 infected Balbc/J and A/J macrophages after stimulation in-vitro with opsonized zymosan (n=18 animals). Where error bars are omitted, standard deviation is < 20 cpm.   I=infected, C=control.

TABLE 4

Free Radical Reaction Products

Host	Time (hrs)	LSF Products	Conjugated Dienes	Dimerized SH Groups	TBA (μg/ml)
		(mg/mg protein ± S.D.)			
Balbc/J	PRE	10.5 ± .08	0.31 ± .07	31.9 ± 6.3	0
	12	24.3 ± 17*	0.36 ± .08	39.8 ± 13.0	0
	24	17.1 ± 3.3‡	0.46 ± .06†	30.6 ± 8.4	0.50 ± .035
	72	16.9 ± 2.9‡	0.49 ± .19*	25.0 ± 1.1*	2.40 ± 1.80
	120	N/A	N/A	N/A	N/A
A/J	PRE	10.3 ± 2.9	0.32 ± .11	32.4 ± 5.2	0
	12	ND	ND	ND	ND
	24	12.5 ± 7.0	0.31 ± .06	31.4 ± 3.7	0
	72	11.8 ± 3.1	0.23 ± .09	29.4 ± 2.6	0
	120	10.3 ± 3.7	0.34 ± .04	21.9 ± 3.5†	0

*p = .05   † p = .005   ‡ p = .0005

Appearance of free radical reaction products in the livers of mice following MHV-3 infection. This is a representative experiment, n = 45 animals (5 per group).

LSF = Lipid Soluble Fluorescence
TBA = Thiobarbituric Acid Positive Material
N/A = not available as mice were dead by this time
ND = not done.

presence of four different free radical reaction products: lipid soluble fluorescent products (LSFP), conjugated dienes (CD), thiobarbituric acid reactive products (TBA) and dimerized SH groups on proteins (SH). The TBA assay measures the formation of malondialdehye (and other aldehydes), and the CD assay the formation of two consecutive double bonds in a lipid. Both are end products of lipid peroxidation. Lipid peroxidation is the oxidative cleavage of polyunsaturated long chain fatty acids in cells and membranes eg. arachidonic acid and phospholipids. LSFP and SH are intermediate products of free radical reactions. LSFP are low molecular weight spontaneously fluorescent products. The SH assay is a measure of the amount of disulphide bonds in proteins under oxidizing conditions (Table 4). The uninfected Balbc/J mice had no increase in lipid peroxidation products. The infected Balbc/J strain mice had a significant increase in three parameters of oxyradical mediated injury. The levels of LSFP, CD, and SH groups were increased at 12 hrs p.i.. LSFP, CD, TBA and SH were increased at 24 hrs p.i. All except the SH groups remained elevated throughout the period of study, while the availability of SH groups at 72 hours had decreased to below normal values. This may reflect utilization of cellular glutathione stores which are a significant source of the main source of free SH groups in the cytoplasm. The A/J mice, both infected and uninfected, had no significant increase in any of the four reaction products up to five days post-infection (Table 4). Because conjugated dienes were representative of cumulative injury, this assay was used in the following protection study.

a) Alteration in conjugated diene formation

Inhibitors of different steps in the pathway of free radical injury were selected. Two iron chelators, which inhibit iron mediated formation of hydroxyl ion were used. Lazeroid U74006F, a potent experimental aminosteroid inhibitor of lipid peroxidation was administered in 1, 10, and 100mg/kg doses. This resulted in conjugated diene levels in the livers of MHV-3 infected mice of 0.072 ± 0.040, 0.091 ± 0.017, and 0.126 ± 0.037

TABLE 5

**Effect of Iron Chelators on
Conjugated Diene Formation and Necrosis in
MHV-3 Infected Balbc/J Mice**

MHV-3 Infection	Treatment Group	Dienes (mg/mg protein ± SD)	% Necrosis
-	—	0.063 ± .05	0
+	—	0.113 ± .05*	48 ± 7.6
-	Laz 1.0 mg	0.055 ± .02	0
+	Laz 1.0 mg	0.072 ± .04	44 ± 8.9
+	Laz 10 mg	0.091 ± .017	28 ± 11*
+	Laz 100 mg	0.126 ± .037	22 ± 16*
	DFO ± Nic	0.054 ± .01	0
+	DFO ± Nic	0.080 ± .05	25 ± 17

MHV-3 = Murine Hepatitis Virus Type 3 (10^4 PFU)
Laz = Lazeroid U74006F from Upjohn (mg/kg/bid)
DFO = Deferoxamine (4.2 mg/day)
Nic = Nicotinamide (10 mg/day)

All mice were sacrificed on Day 3 p.i., n=10 per group.

* = significant at $p \leq 0.05$

mg/mg protein $\pm$ SD, respectively, a reduction of 36, 19 and 0 % by comparison with the controls. Although none of these three changes were significant at the level of $p<.05$, compared with the untreated mice, the 1mg/kg dose produced the greatest reduction in conjugated diene levels. The second iron chelator was deferoxamine (DFO), which was used in combination with nicotinamide to replete the cellular stores of NADP depleted by the DNA repair enzymes which are activated with the onset of injury. This combination given without viral infection lowered the amount of conjugated dienes formed to 13% below that in the uninfected mice, an effect comparable to U74006F treatment (Table 5). In the MHV-3 infected mice, DFO plus nicotinamide reduced conjugated diene formation by 29%, when compared to the untreated virally infected mice. A/J mice did not have a significant increase in conjugated diene formation.

The ability of an additional group of agents (superoxide dismutase, catalase, diphenyl phenylenediamine (DPPD), and CV3988, a PAF antagonist) to protect against conjugated diene formation was tested. None of the treatments significantly decreased conjugated diene formation (Tables 6,7), although DPPD came the closest to doing so.

Both SOD/catalase and DPPD caused a slight (but not quite significant) decrease in conjugated dienes in the normal, uninfected mice, while CV-3988 had no effect. In fact, the magnitude of the decrease of conjugated dienes induced by SOD/catalase and DPPD was similar in both normal and MHV-3 infected mice (Table 6).

b) Alterations in necrosis

Biopsies of liver tissue from both MHV-3 infected and uninfected groups of Balbc/J mice were examined at the time of sacrifice. There was no necrosis in the control group or any treatment group that did not also receive MHV-3 . Lazeroid U74006F treatment in doses of 1,10, and 100 mg/kg/bid resulted in decreases in necrosis from $48 \pm 8\%$ group in the receiving virus only to 44 ± 9, 28 ± 11, and $22 \pm 16\%$, respectively in the Lazeroid

TABLE 6

Effect of Free Radical Inhibitors on
Conjugated Diene Formation and Necrosis in
MHV-3 Infected Mouse Livers

MHV-3 Infection	Treatment Group	Dienes (mg/mg protein ± SD)	% Necrosis
-	—	0.131 ± .038	0
+	—	0.199 ± .036	18.6 ± 7.5*
-	S/C	0.102 ± 0.23	0
+	S/C	0.175 ± .071	19.6 ± 21.4
-	DPPD	0.103 ± .025	0
+	DPPD	0.163 ± .013	6.6 ± 5.0*

MHV-3 = Murine Hepatitis Virus Type 3
Dienes = Conjugated Dienes
S/C = Superoxide Dismutase (8 mg/kg/bid) + Catalase (8 mg/kg/bid)
DPPD = Diphenyl Phenylenediamine (600 mg/kg/bid)

All mice were sacrificed on Day 3 p.i., n=5 per group.

* = significant at $p \leq 0.05$

TABLE 7

Effect of a PAF Antagonist in Combination with Free Radical Inhibitors on Conjugated Dienes and Necrosis in MHV-3 Infected Mouse Livers

MHV-3 Infection	Treatment Group	Dienes (mg/mg protein ± SD)	% Necrosis
—	—	0.131 ± .038	0
+	—	0.199 ± .036	18.6 ± 7.5*
—	CV3988	0.132 ± .043	0
+	CV3988	0.173 ± .057	12.4 ± 12.9
+	CV3988 + DPPD	0.174 ± .078	5.9 ± 9.2*
+	CV3988 + S/C	0.151 ± .037	3.4 ± 5.7*

MHV-3 = Murine Hepatitis Virus Type 3
CV3988 = Platelet Activating Factor Antagonist (10 mg/kg/bid)
Dienes = Conjugated Dienes
S/C = Superoxide Dismutase (8 mg/kg/bid) + Catalase (8 mg/kg/bid)
DPPD = Diphenyl Phenylenediamine (600 mg/kg/bid)

All mice were sacrificed on Day 3 p.i., n=5 per group.

* = significant at $p \leq 0.05$

MHV-3

C

Ab + C

EC

Other Potential
Mechanisms of Injury

IL-1
IFN
TNF
Others

KC

Activation of
A.A. pathway

PCA   PAF O_2^- + H_2O_2

A.A.
Products
esp. PGD2

Fibrin   Pl aggregation   OH⁻

activation of
LOase
COase

Coagulation

Lipid peroxidation
Protein
DNA

Ischemic
Injury

Hepatocellular necrosis
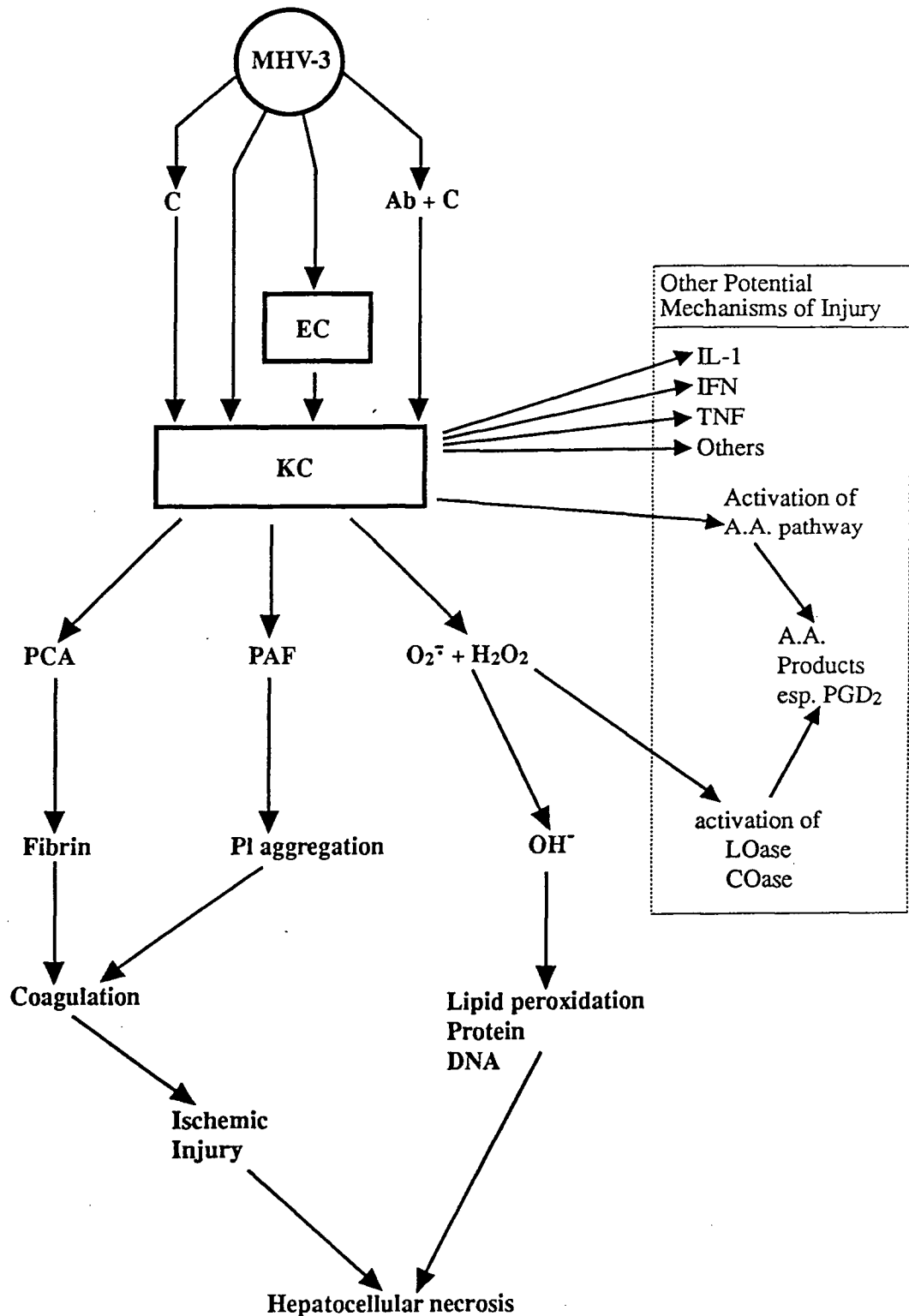
Figure 22   Schematic of Mechanism of Injury.
MHV-3 = Murine Hepatitis Virus Type 3. C = Complement. Ab = Antibody.
EC = Endothelial Cell. KC = Kupffer Cell. PCA = Procoagulant Activity.
PAF = Platelet Activating Factor. Pl = Platelet. IL-1 = Interleukin 1.
IFN = Interferon. TNF = Tumor Necrosis Factor. A.A. = Arachidonic Acid Pathway.
PGD2 = Prostaglandin D2. LOase = Lipooxygenase. COase = Cyclooxygenase.

treated groups. From these results it became clear that increasing the dose of Lazeroid U74006F to greater than 1mg/kg was effective in reducing the amount of necrosis due to the virus, while it had no effect on conjugated diene formation . Desferrioxamine + nicotinamide reduced the necrosis to 27% of that seen without treatment. All values for necrosis are included in Table 5.

In the second inhibitor study superoxide dismutase (SOD) + catalase (C) had no effect on liver necrosis. The platelet activating factor antagonist (CV-3988) was chosen to see if U74006F to greater than 1mg/kg was not effective in reducing the amount of necrosis due to the virus. Desferrioxamine + nicotinamide reduced the necrosis to 27% of that seen without treatment. All values for necrosis are included in Table 5.

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In the second inhibitor study superoxide dismutase (SOD) + catalase (C) had no effect on liver necrosis. The platelet activating factor antagonist (CV-3988) was chosen to see if inhibition of platelet aggregation alone, or in combination with free radical inhibitors, could inhibit liver necrosis. CV3988 alone did not have a significant effect on liver necrosis. CV-3988 was most effective in inhibiting necrosis when given in combination with SOD/Catalase (Table 7). DPPD alone (Table 6) or the combination of SOD/Catalase plus CV-3988 were both highly effective at reducing necrosis (Table 7), p <.005. DPPD plus CV-3988 was no better than DPPD alone. There was no evidence of any liver necrosis or other pathology in the A/J mice at any time post-infection.

DISCUSSION

MHV-3 as a model of viral hepatitis An understanding of the early events post viral infection which led to the induction of hepatocellular necrosis is vital for the development of effective clinical strategies in the treatment of viral hepatitis. Moreover, an elucidation of these mechanisms impacts our entire understanding of viral injury. Despite the extensive progress in medical science in the area of bacterial infections, outside of immunization there are very few strategies available to cope with viral infections.

In these experiments we have been greatly assisted by the availability of genetically distinct inbred mouse strains that allow the investigator the choice of predictably observing an acute fulminant hepatitis (Balbc/J), chronic granulomatous hepatitis (C3HebFe/J) or a fully resistant strain (A/J).

Although there has been much discussion of viral pathology in the literature, there has been less emphasis on pathogenesis. In the experiments described here histological, biophysical and immunological techniques have been used to define the processes and cells involved. This murine model of hepatitis uses a well defined member of the coronavirus family as the inducer of hepatitis. It is particularly suitable as a model of human hepatitis as the lesions induced are both histologically and ultrastructurally indistinguishable from human hepatitis, with the exception that in humans one cannot yet predict an individual human's response to the virus, whether acute hepatitis with recovery, or death, or possibly a prolonged chronic aggressive course. This work concentrates on the acute fulminant hepatitis induced by MHV-3 in Balbc/J mice. This is, perhaps, a worst case situation, as Balbc/J mice uniformly die of the disease. Any intervention, then, that can change an invariably fatal course to one of survival bears

considerable promise of providing insight into the critical processes involved. Furthermore such a treatment might be expected to have significantly more benefit in the case of a non-fulminant disease, which represents the major portion of human viral illness.

Histological and Ultrastructural Findings during MHV-3 Infection Light microscopic findings in the livers of mice infected with MHV-3 have been previously documented (McNaughton and Patterson 1980, Levy et al 1983). In brief, by 24 hours post-infection focal areas of hepatocellular necrosis were seen, accompanied by a sparse polymorphonuclear leukocytic (PMN) and more marked monocytic infiltrate. In the fulminant hepatitis seen in susceptible Balbc/J mice, the areas of necrosis gradually enlarged until near confluence at the time of death 5-7 days post-infection. This was in sharp contrast to the heavy infiltration of lymphocytes, and minimal necrosis seen in the chronic C3H/ebFeJ model (MacPhee et al 1985). No pathological changes are evident in the fully resistant A/J strain (Levy et al 1983).

The ultrastructural findings at 12 hours post-infection showed that significant changes had already occurred. Occasional sinusoids were blocked with an accumulation of cells. Individual hepatocytes were undergoing various degrees of cell degeneration, with cell swelling, decreased cytoplasmic electron density, dilation of the rough and smooth endoplasmic reticulum and loss of mitochondrial matrix granules, indicating breakdown of intramitochondrial calcium stores. In extreme cases the hepatocellular membrane was no longer intact and the sinusoidal lining completely absent. In these cases no endothelial or Kupffer cells were seen overlying the hepatocytes, presumably having previously become detached from the hepatocytes. In short, a lethal insult had already been delivered to both sinusoidal and parenchymal cells. The appearance of the roughly spherical unperfused areas by scanning electron microscopy is consistant with the onset

of injury at a point source, with gradual cell to cell spread in all three dimensions. The ultrastructural changes at 2-3 days indicate more extensive areas of cell death, and the uptake of cell debris by remaining Kupffer cells. The absence of viral particles in normal or dying hepatocytes in the time under intense study (days 1-3), indicate that viral replication could not have been widespread in hepatocytes up to this point.

The changes seen in the hepatocytes, if viral particles are indeed absent, must be due to an external factor which gives a lethal hit very early post-infection. The mitochondrial changes and the appearance of extensive membranous whorls are similar to those seen in carbon tetrachloride induced liver damage, and to those due to ischemic injury (Recknagel et al 1973, Phillips et al 1987c). Major features of this type of injury include vesiculation and dilatation of smooth endoplasmic reticulum, fragmentation and detachment of ribosomes, and dilatation of intracisternal spaces of the rough endoplasmic reticulum. Membranous whorls are frequently seen in the cytoplasm, along with areas of increased matrical density in the mitochondria, an increased number of lysosomes (often associated with areas of focal cytoplasmic degeneration), an increase in mainly macrovesicular fat, dilatation of the bile canaliculi with blunting of microvilli and, later, changes in the nucleus such as peripheral condensation of the chromatin. In short, these changes produce the classic appearance of ballooning degeneration of the hepatocytes. Coagulative necrosis is the hallmark of irreversible cell injury , and is characterized by both nuclear (chromatin clumping, or disintegration) and cytoplasmic changes (e.g eosinophilia, increased intracellular volume, mitochondrial swelling and calcification) (Farber 1982). The mechanism of cell death is not entirely clear. However, it appears that changes in calcium influx (reflecting changes in membrane permeability) may play an important role (Trump and Bejezesky 1985). The onset of liver microsomal changes in CCL_4 toxicity occurs within minutes of intragastric administration. Recknagel and Glende (1973) have demonstrated greater than half-maximal changes in conjugated

dienes from rat liver microsomes at five minutes, protein synthesis inhibition by 20 minutes, and mitochondrial lipid peroxidation by two hours after administration.

The appearance of acidophilic bodies and of other degenerating hepatocytes, as in human hepatitis, is also seen in MHV-3 hepatitis. There are no differences at the electron microscopic level between the effects of hepatitis A, B, non-A non-B, Herpes, Adeno, Echo and HIV viruses (Phillips et al 1987b, Inoue et al 1987). They all cause ballooning degeneration with pale, swollen cytoplasm, irregularly dilated smooth and rough endoplasmic reticulum, and large mitochondria with patchy areas of high density, paracrystalline arrays (considered by Trump et al 1965a to be uricase), and whorled lamellae. There was little evidence in this viral model of an increase in lipids, where present it was restricted to occasional cells and was not nearly as prominent as is seen in Reye Syndrome (Shubert et al 1973), or in starvation. Fatty change is seen in non-A non-B hepatitis in man; however our studies are limited to the first three days post-infection, compared to several weeks post infection in the case of human hepatitis. This spectrum of changes is also seen in radiation injury and with certain drugs and toxins (Phillips et al 1987c,d and Tanikawa 1979).

The mechanism of induction of these changes is unknown (Phillips et al 1987a-d). One mechanism which has been proposed is that lysosomal rupture and release of enzymes following initial cell injury leads to further injury and cell death, but this theory has been largely discounted, as it appears lysosomes are stable until after the process of necrosis becomes irreversible (Hawkins et al 1972).

The significance of the sharply circumscribed and gradually increasing areas of necrosis became clearer when seen in the light of the immunofluorescent cell studies which enabled us to identify the constituents of the monocytic infiltrate. Although a strong

component of activated T cells might be viewed as the cytotoxic agent because of the ability of T-cells to become specifically activated against viral targets, the conspicuous absence of all T-cell subsets in the hepatic parenchyma makes hepatocellular necrosis via T-cell mediated cytotoxicity very unlikely. The identification of the infiltrating mononuclear cells as Mac 1 positive (Mac 1+) indicated a monocyte/macrophage lineage. The question of whether these were important in the induction of necrosis or possibly accumulated secondarily as a result of injury was approached in several ways, including ultrastructural studies. The peak increase of monocyte accumulation occurred after 48 hours post-infection. Therefore,if Mac 1+ cells were involved in the initiation of hepatocellular necrosis, those belonging to the resident population must be primarily responsible. Immunofluorescent studies also showed large numbers of Mac 1+ mononuclear cells around the outside of the lesions at days 2 and 3; these could be the result of Kupffer cell migration or monocyte influx from the periphal blood, or both.

The nature of the injury, the rapid time course, and the presence of Mac 1+ cells suggest that the hepatocellular necrosis might be due to release of a toxic factor after uptake of the virus by sinusoidal cells. The ultrastructural evidence suggests that an injury to the cell membrane was responsible for the rapid onset of cell death. In the studies reported here the presence of extensive lipid peroxidation as early as 12 hours post-infection was confirmed by biochemical studies in which the different assays indicated an increase in free radical reaction products.

It has been previously observed that MHV is taken up by, and replicates first within, the Kupffer cells of the liver (Bang and Warwick 1960, McNaughton and Patterson 1980). It is not surprising that MHV-3 is taken up preferentially by the liver, since this organ contains up to 90% of the reticuloendothelial system of the body (Jones and Summerfield 1982). Kupffer cells can remove 90% of particulate matter from the blood on first pass

through the liver, and endothelial cells also have the ability to take up small particles (Hampton 1958, McCuskey et al 1986). In mouse liver, the distinction between endothelial and Kupffer cells is much less clear than in humans or rats. In rats, only Kupffer cells have peroxidase activity, whereas in mice 50-60% of apparent endothelial cells are also strongly peroxidase positive (Fahimi 1982). Kupffer cells also have peroxidase positivity in the rough endoplasmic reticulum and the nuclear membrane (Geerts et al 1988, Hightower et al 1987, Muro et al 1987). The amount of positivity increases significantly after Kupffer cells have been activated. Kupffer cells can serve as antigen presenting cells (Reichman et al 1979). Morphological distinction in the mouse is difficult as intermediate forms with various numbers of lysosomes and phagocytosed material exist. Endothelial cells also lie in the sinusoids, have processes, phagocytose (Steffan et al 1986), and present antigen (Richman 1979); therefore, a clear separation of Kupffer cells from endothelial cells is not possible by these criteria. Both types of cells show changes such as irregularity of nuclear outline and cytoplasm when activated. Studies in this model with small particles such as india ink also show a diffuse uptake of carbon particles along the endothelium that is easily seen in vivo.

Previous work by Ruebner and co-workers (Reubner and Miyai 1961, Ruebner et al 1967) and Boss and Jones (1963) has shown that ultrastructural changes occur first in Kupffer cells, and are followed by changes in endothelial cells in the Swiss mouse infected with MHV-3. Similarly, changes have been seen in Kupffer cells prior to hepatocytes in Coxsackie B1 hepatitis (Burch et al 1973), and Frog Virus Type 3 (FV-3) infection of mice (Kim et al 1983). The results of these present ultrastructural and scanning electron microscopic studies are consistent with the previous observations made in this model indicating primary infection and changes in the sinusoidal lining cells which precede those in hepatocytes.

The appearance of discrete lesions at 24-48 hours of a fairly uniform size (diameter of 83 $\pm$ 26um , mean $\pm$ SD) when seen by SEM (MacPhee et al 1988), also seen in vivo (Levy et al 1983, MacPhee et al 1985), show that the damage is initiated almost simultaneously throughout the liver, but occurs in a select cell population. Spherical lesions develop over the first 2-3 days p.i., gradually involving many cells. Their spherical appearance suggests enlargement by cell to cell spread in three dimensions, and their distribution is consistent with the scattered nature of the initial single cell lesions. The actual number of cells infected, and hence the number of lesions, would depend on whether or not the number of live viral particles in the innoculum was a limiting factor. These observations suggest that Kupffer cells are the site of origin, and that they also play an important role in the initiation of the hepatocellular changes.

In a carbon tetrachoride model of liver damage in mice, carbon laden Kuppfer cells were found in larger numbers at the periphery of the damaged zones of the liver, and were presumed to have migrated there, as the numbers of cells in the normal areas were comparatively diminished (Parry 1978). Carbon loading in rats by tail vein injection of ink leads to uptake within 10 min by periportal Kupffer cells, and later in zone 3 by Kupffer and endothelial cells as well as in spleen and bone marrow macrophages. Days later, carbon particles were found in regional lymph nodes of the liver; first in the interfollicular areas and paracortex and two weeks later in the medullary cords (Hardonk et al 1986). During our in vivo microcirculatory studies we have seen certain large granular cells moving upstream in vivo, a property which to my knowledge has not before been observed in action. Such cell movement may represent normal movement of resident (but not fixed) tissue macrophages. Kupffer cell proliferation has also been documented under certain stimuli. An additional potential source of Mac 1+ cells is an influx of new monocytes from the bloodstream, which were selectively drawn to the area,

or arrested there by the early microcirculatory changes in the area. Of course, the Mac 1+ cells may represent a combination of these sources.

Our studies indicated that Mac 1+ mononuclear (monocyte/macrophage lineage) cells accounted for almost the entire cell infiltrate, with the exception of a few PMNs. No T-cells were present and functional inhibition studies using cyclosporin A (CsA), which is known to inhibit activation of T-helper cells (Feurer et al 1988), showed no significant protective effect. It therefore seems improbable that T-cell mediated cytotoxicity is an important mechanism of hepatocellular injury. In addition, there was no infiltrate present in the resistant A/J strain which, if present, could be construed to represent protective or suppressor cells.

The lymphocyte proliferation assays (Fig 17) have shown that although both strains are capable of responding to MHV-3 in vitro, the response of both A/J and Balbc/J splenocytes to MHV-3 is greater when the antigen presenting cell is an A/J macrophage, indicating that the difference lies not with the T cell but at the macrophage level. A/J mice are H-2^a , and Balbc/J mice H-2^d, and share common Class II antigens within the K region. The proliferation of splenocytes after exposure to MHV-3 antigen on foreign macrophages differing at the major histocompatibility complex (H-2) is intriguing . Possibly, this may be due to proliferation of a subgroup of T cytotoxic lymphocytes (CD8+) which respond to Class I antigens, rather than proliferation of T helper cells, which require compatible class II antigens (Klein 1986). Another possibility is that primed cells may exist due to prior exposure of the mice to a non-virulent MHV (as indicated by the antibody studies); this may account for moderately high counts with a relatively short incubation time (48 hours). A third possibility is that the virally altered cell membranes mimic histocompatibility antigens in such a way as to induce a mixed lymphocyte reaction, as proposed by Greaves et al (1979). The reason for the decreased

response of the Balb/cJ mice is not certain. It could be due to a defect is antigen processsing or presentation of viral antigen to T-cells, or to an impairment of co-stimulatory activity related, for example, to a decrease in production of interleukin -1, or to direct inhibition of the T cell response by the release of immunosuppressive products such as prostaglandins or free radicals from the activated macrophage.

MHV-3 infection has also been shown to have an effect on the humoral immune reponse in susceptible mice. A decrease in all immunoglobulin classes was seen in the semi-susceptible C3H/HeJ strain by Day 20-30 p.i., and the immunoglobulin levels did not recover until Day 215 p.i. (Le Roy et al 1982). LeRoy suggested that depression of lymphocyte proliferation may be involved, as MHV-3 infection decreased Con A stimulated lymphocyte proliferation of allogeneic cells. In contrast, in the resistant A/J strain an effective neutralizing antibody response develops and viral titres are decreased by Day 3 in the serum, and by Day 7 p.i. in the liver (Levy et al 1983).

In vivo microcirculatory studies One of the advantages of in-vivo microcirculatory studies is the ability to observe flow over a period of hours, provided the animal is properly supported. Microcirculatory changes occurred early (within 8-12 hours) following infection with MHV-3 (Levy et al 1983, MacPhee et al 1985, McCuskey et al 1986). Focal slowing of blood flow and blockage of sinusoids precedes the appearance of lesions as seen by light microscopy. Areas consisting of as few as 1 or 2 hepatocytes could be distinguished under high magnification. Such cells appeared to be swollen and the red cell velocity could be seen to decrease as the cells entered the area, and increase when past the edematous cells. At a certain point flow could be seen to cease through the area, and blood was then shunted into neighbouring sinusoids. The extensive sinusoidal interconnections ensure that only the focal area of injury was deprived of its blood supply. As the lesions developed, the areas without flow were seen to be roughly

spherical in shape and had well defined borders. This shape was confirmed by the scanning electron micrographs of MHV-3 infected livers. The sinusoids within the lesions appear to be blocked as opposed to attenuated, as the microcorrosion casts of sinusoids bordering the lesions ended abruptly with concave impressions (MacPhee et al 1988). It appears then, that the flow alterations were limited to areas where cells had previously been damaged. The blockage of the sinusoid could be due to a gradual narrowing of the sinusoids by ballooning hepatocytes until red cell flow was prevented, and/or the damage to endothelial or Kupffer cells may have led to exposure of basement membrane or release of procoagulant activity, platelet activating factor, or other factors which may activate coagulation locally, inducing the formation of a microthrombus.

The normal diameter of the murine sinusoids is sufficient to allow free passage of both leucocytes and erthrocytes (rbc), although the white cells frequently can be seen to move slower than rbc's through sinusoidal junctions. This is a result of their relatively limited deformability. In the normal liver certain white cells were also seen in-vivo to move upstream against red cell flow. To my knowledge, neither the ability of macrophages to move upstream, nor the speed of macrophage migration in the liver microvasculature, has previously been recorded. However, co-workers from the same microcirculatory laboratory have recently observed similar cell movement in the spleen (EE Schmidt et al in press).

Parry (1978) stated that "free Kupffer cells are then carried by the bloodstream to the centrilobular zone, where strong chemotactic factors, probably combined with disturbed tissue architecture, bring about their arrest." Movement of macrophages in a rabbit ear chamber model was recorded by Cliff (1966), with the highest speed of migration being 40um/day. The speed of migration of macrophages of the spleen was up to 3 orders of magnitude higher, i.e. 48um/min. The ability of these cells to move against a normal rbc

velocity of 69um/sec suggests considerable adhesive and propulsive ability. Macrophages have also been observed in-vivo (in our studies) to advance and retract, and move from one side of a sinusoid to another, apparently always keeping in contact with one cell wall. These observations make it possible to envisage macrophage migration to a site upstream of their original location, possibly in response to a chemotactic stimulus. It is possible that the ability to migrate upstream is a general property of at least certain classes of what have formerly been known as 'fixed tissue macrophages'.

The Theory of Direct Viral Cytopathicity Examined The first question to be addressed in discussing the pathogenesis of any viral injury is the role of direct cytopathic effect. Any potential mechanism of injury in this model must account for the significantly low LD_{50} for this virus in the susceptible Balbc/J mice of 5 PFU of MHV-3. Even at Day 5, when the liver was almost totally necrotic , the maximum viral titres were in the order of 10^7 PFU/gm liver (MacPhee et al 1985). As this represents only 1 PFU/10-100 liver cells (there are approximately 10^{8-9} cells per g liver), it would seem unlikely on this basis alone that direct viral cytopathicity can account for the massive hepatocellular necrosis. The virus is also likely to be located mainly in the cells in which it replicated. Indeed, electron microscopic studies (Bang and Warwick 1960) have shown that endothelial and Kupffer cells can be damaged very early post-infection (12 hours), without any evidence of any viral replication in hepatocytes. These studies have also shown that many severely damaged or necrotic cells have no virus present.

Previous studies (MacPhee et al 1985) have shown that MHV-3 antigen is mainly located (using indirect immunofluorescence) in sinusoidal lining cells at 24 hours post-infection. At seven days, coincident with neutralizing antibody formation, sinusoidal lining cells are mainly negative, and only small groups of a few hepatocytes show positivity. The

replication of MHV-3 in the fully resistant A/J strain to between 10^6-10^7 PFU/g, similar to levels seen in the C3Heb/FeJ mice, some of which develop acute and some chronic hepatitis, emphasizes that viral replication alone is not the decisive factor in the development of liver necrosis.

It has been assumed in the past, in the case of non-cytopathic viruses that the injury is immune mediated, i.e. due to recognition of foreign viral antigens or altered host antigens on the surface of virally infected cells by cytoxic T-cells, with resultant destruction of the cells. The mechanism by which this occurs has not been is not yet understood (Koff and Galambos 1987, Phillips et al 1987 and Zuckerman 1987). The role of cell-mediated immunity (CMI) in the case of hepatitis B has been investigated; and although there is a temporal relationship between the appearance of immune cells and host cell destruction, it seems likely that this is not the cause of the cell death, but is more important in the removal of already damaged cells (Koff and Galambos 1987, Zuckerman 1987, Hirsch 1984).

Viruses can injure cells by a variety of mechanisms. One that sheds an interesting light on the role of Kupffer cells in this model is injury via toxic factors as in Frog Virus 3 (FV-3) infection. This virus does not replicate at 37°C and induces a fulminant hepatitis even when the virus is solubilized (Gut et al 1981). In vivo this virus is taken up by the reticuloendothelial system in all organs, but the damage due to the virus is seen only in the liver. It appears then that the virus itself then is not solely responsible for the hepatocellular necrosis. The mechanism seems to be the uptake of FV-3 from the blood by the Kupffer cells, followed by the lysis of infected Kupffer and endothelial cells, with the release of unknown factors that inhibit synthesis of certain essential hepatocellular compounds (Gut et al 1981). Interestingly, when FV-3 is given to MHV-3 resistant A/J mice, the A/J's become susceptible to MHV-3 (Pereira et al 1984). Other

authors have pointed out the protective role of Kupffer cells in viral infections (Kirn et al 1982a,b,and Taguchi et al 1983). FV-3 infected A/J mice develope MHV-3 damage similar to that seen in MHV-3 infection of susceptible strains, ie numerous foci of hepatocellular necrosis progressing to fulminant hepatitis.

In other experiments Steffan and Kirn (1979) showed that FV-3 treatment of Swiss mice also led to a decrease in resistance to vaccinia virus. Swiss mice are normally resistant to the non-hepatotrophic vaccinia virus. As previously mentioned, viruses can also cause damage when expression of viral antigens on infected cells induces T-cell-mediated cytotoxicity. Ultrastructural studies in the FV-3 model also show that the first liver lesions occur in the Kupffer and endothelial cells (Kirn et al 1983), and are visible as early as 1 hour post-infection . Hepatocellular nuclear lesions are visible at about 3-5 hours post-infection and cytoplasmic changes at 8-14 hours p.i. This is similar to that seen in our MHV-3 ultrastructural studies where cytoplasmic changes are well-developed in individual hepatocytes by 12 hr p.i. Frequently, no endothelial or Kupffer cells were seen overlying these cells, possibly indicating prior lysis and detachment from the underlying hepatocytes. In both models, whole viral particles were never observed in the hepatocytes. Kirn et al (1983) suggested that since the virus itself does not induce the hepatocellular changes seen in the FV-3 model, primary damage to Kupffer cells may result in secondary endotoxin damage to hepatocytes, when the Kupffer cells are no longer able to cope with its normal removal. McCuskey et al (1986) during in-vivo studies of FV-3 reported that alterations in the hepatic microcirculation were detected as early as 20 min post-infection, with extensive congestion of sinusoids due to aggregates of platelets, detached Kupffer cells and swollen endothelial cells by 3 hours. They also suggested that since the changes seen were similar to those after endotoxin, that endotoxin may be responsible. In addition, they postulated an additional effect due to

hypoxia from sinusoidal blockage. This emphasizes the possiblity that different mechanisms may be involved in the death of each cell type.

The response to endotoxin is one of the most extensively studied models of injury involving mouse macrophages. As a result of these studies it has become apparent that the absolute numbers of Kupffer cells present in the liver of different mouse strains has a profound effect on the outcome of endotoxin administration. C3H/HeJ mice have both a low response to endotoxin and low numbers of phagocytes in the liver, as well as a deficiency in lysosomal enzymes, whereas the C3HeB/FeJ mice have 58% more Kupffer cells and a high response to endotoxin. Interestingly, this strain develops an acute hepatitis, followed by either resolution or chronic granulomatous disease (MacPhee et al 1985), an intermediate response to MHV-3 between that seen in the susceptible Balbc/J and that in the resistant A/J mice. C3H/HeJ mice also have a low response to Frog Virus 3, in contrast to the C3HeB/FeJ strain which develops severe microcirculatory disturbances and toxic hepatitis.

JHMV (or MHV-4) is closely related to the MHV-3 strain of mouse hepatitis virus. It is a neurotropic virus which induces selective demyelination in the brain, and has been widely used as a model of multiple sclerosis and postinfectious encephalomyelitis. Recent studies have shown that it exhibits a selective trophism for microglial cells (and astrocytes) , which are the local tissue macrophages in the brain (Massa et al 1986). These authors have stated that cytopathic effects spread by cell-to cell fusion, which leads to lesions remarkably similiar to the type of lesions seen in the liver after MHV-3 infection, most clearly visible in the roughly spherical deficits seen by scanning electron microscopy of the liver casts. Once an hepatocyte is damaged , it may in turn release substances which injure cells on its borders with subsequent spread of the damage in three dimensions. The mechanism of this is unknown, but is not likely to be due

primarily to ischemic damage from blocked vessels as the damaged areas would then tend to be the shape of the area supplied by that vessel.

Role of macrophages and Kupffer cells   There are two types of mononuclear phagocytes which could be important mediators of tissue injury in this model: the liver-specific Kupffer cells, and the peripheral blood monocytes.   The appearance of monocyte procoagulant activity (mPCA) early in the course of infection in the susceptible mice as previously reported (MacPhee et al 1985b, Dindzans et al 1985), and the protective effect of the defibrinating agent Ancrod (a derivative of a snake venom) (MacPhee and Levy 1985) also support macrophage activation as important in the pathogenesis of the hepatocellular necrosis. MHV-3 is known to be picked up first by the liver Kupffer and endothelial cells and replicate there (Boss and Jones 1963).   SEM studies have also shown that the necrotic areas are very uniform in size at any given time post-infection, indicating probable simultaneous initiation.   The lesions are nearly spherical in shape, which suggests some form of cell-cell spread in three dimensions (MacPhee et al 1988). The spherical shape of the necrotic areas argues against flow blockage in the higher order vessels as the primary cause of necrosis (i.e. due to ischemia) as this would result in a wedge-shaped lesion due to the nature of the branching of the afferent hepatic vasculature.   However, local blockage in an individual sinusoid due to microthrombi or cell swelling, or blockage of a sinusoid with cell debris and secondary ischemia of local hepatocytes, could significantly add to the degree of damage. In-vivo observations however, frequently show hepatocellular edema of a few cells (as in Fig 2a) in areas where flow is not yet completely occluded, only slowed, and therefore it is not likely that reduced flow alone would cause sufficient ischemia to induce hepatocellular edema. Edema usually indicates serious damage to cell membranes with loss of intracellular homeostasis.

Monononuclear phagocytes, like polymorphonuclear leukocytes (PMN's), are capable of producing oxygen free radicals (OR) and excited oxygen species in response to a phagocytic stimulus. The scarcity of PMN's seen in the liver parenchyma after MHV-3 infection and the increase in numbers of mononuclear cells, make mononuclear cells the most likely source of oxygen radicals, such as H_2O_2, $\cdot OH$, and $O_2 \cdot$. The half-life of free radicals ranges from 10^{-9} sec for $OH \cdot$ to microseconds for $O_2 \cdot$, thereby limiting the distance over which they can diffuse. Damage to hepatocytes would therefore require the source of free radicals to be very close to the target tissue, which is the case for the overlying Kupffer cell. Our in vitro studies have demonstrated that peritoneal macrophages from the resistant A/J strain mice do not respond with enhanced chemiluminesence upon infection with MHV-3 or opsonized zymosan. A strain specific defect in the macrophage could account for the lack of hepatocellular necrosis in the A/J strain, despite their ability to replicate this virus (MacPhee et al 1985). Hirano and Reubner (1966) have also recorded significant viral titres from the spleens of CFW mice, which did not exhibit necrosis. The ability of macrophages to chemiluminesce after stimulation, an indicator of free radical release, differs between organs and species. Cells from newborn mice (Sonderer et al 1987) can be stimulated to chemiluminesce by PMA, zymosan, or antibody coated red cells. Fujiwara et al (1989) found that rat Kupffer cells in-vivo, when activated with PMA, released superoxide in zones 1 and 2 of the liver, which could be prevented using SOD inhibitors.

The increase in free radical production in MHV-3 infected Balbc/J macrophages may not only have a role in tissue injury, but also in inhibiting lymphocyte proliferation and clonal expansion of antibody producing cells (LeRay et al 1982). This has been noted in other models such as graft versus host disease (Goldin and Keisari 1986), by alveolar macrophages (Warren et al 1987) and bacterial killing by phagocytes (Babior 1978). Peterhans (et al 1988) have studied in vitro the interactions of several other viruses

(measles, influenza, sendai) with different cell types (eosinophils, neutrophils, monocytes and macrophages) and concluded that there is specificity with regard to both virus and cell type; i.e. sendai virus stimulates release of reactive oxygen species (ROS) from neutrophils and monocytes but not eosinophils and glial cells. In addition, the peak time of ROS release differs from virus to virus and cell type to cell type.

It is possible that the failure of the macrophages from A/J mice to chemiluminesce following infection is related to a virus-specific difference in virus-cell interactions. The chemiluminescence of human monocyte/macrophages (Jungi and Peterhans 1988) appears to be triggered by two pathways, only one of which is activated by Sendai Virus. In addition, in this model when peripheral blood monocytes were allowed to mature in-vitro, they lost the ability to respond to this virus. It is also interesting to recall that specific antibody in the presence of antigen can bind to Fc receptors and induce chemiluminesce (Peterhans et al 1983), in the light of the likely presence of preformed cross-reactive antibody in these mice. A/J mouse macrophages may lack receptors for MHV-3 which are linked to activation of chemiluminesce. However, Balbc/J macrophages infected in vivo with MHV-3 for 48 hr and then removed and stimulated with zymosan responded with greater chemiluminescence than macrophages isolated and infected in vitro, possibly reflecting prior macrophage activation in vivo, with a resultant increase in peroxidase activity.

New data in this model (MacPhee and Keown 1989) have shown that there is indeed free radical induced lipid peroxidation in the livers of susceptible mice. Lipid peroxidation, as attested to by an increase in lipid soluble fluorescent products and conjugated dienes, was present in the livers of MHV-3 infected Balbc/J mice by 12 hrs p.i. Our data have also shown this was associated with an increase in dimerized SH groups. This may reflect oxidation of glutathione (Brigelius et al 1983), the most readily available intra-

cellular free radical scavenger, as well as oxidation of other liver proteins. The decrease in dimerized SH-groups at both 72 and 120 hours p.i. to well below normal, is consistent with oxidation of glutathione (which is more readily released from the cell), followed by depletion of glutathione stores. The late (24 hr p.i.) appearance of significant thiobarbituric acid positive material may relect the fact that this assay measures secondary products of lipid peroxidation, or that only at this point is cell necrosis occurring to a degree that overwhelms intra- and extracellular scavenging mechanisms.

Natural Killer Cells Since the discovery of spontaneously cytotoxic or natural killer cells in isolated lymphocyte preparations, much work has been done to define their function in-vivo. In mice these cells have been found to destroy exogenously administered tumor cells in vivo, although their effectiveness against spontaneously arising cells is controversial (Trinchieri and Perussia 1984). Infection with several viruses including CMV has been shown to induce both IFN and NK activity in-vivo in mice. Some strains of mice have a genetically defined "low" or "high" level of NK activity. Linkage studies suggest that several genes are involved, at least one of which is H-2 linked and which involves more than 1 chromosome (Petranyi et al 1975). In these experiments A strain mice had the lowest NK activity of the strains tested. NK cells are normally Thy 1+ and asialo Gm1+ in mice (Piguet et al 1987). Schindler et al (1982) using MHV-3, investigated the induction of NK activity after infection in C57Bl6/J/BOM and A/J /BOM mice at 10-13 weeks of age. C57Bl6 mice are fully susceptible to MHV-3, as are Balbc/J mice . Schindler found that the susceptible mice had a high level of NK activity and the resistant a low activity, which is consistant with previous results using A/J mice. This was not true for HSV infection, which did not induce IFN in A/J mice, but did in the HSV resistant C57Bl6 mice. They concluded that although NK cells may be important in protection against certain viral infections, they are

not in MHV-3 infection. They also demonstrated that the ability of a virus to induce NK cytotoxicity does not correlate with resistance to that virus.

There are a number of other arguments against NK cells as effectors in the hepatocellular necrosis seen in MHV-3 infection. The immunofluorescent studies did not show any Thy 1+ cells in the liver parenchyma up to three days post-infection, when necrosis was well developed. This makes it unlikely NK cells, which are Thy 1+, were present. Secondly , the aggregate of evidence suggests that NK cells do not use toxic oxygen products as agents of cell lysis (Storkus and Dawson 1986, Bishop et al 1987, Van Kessel et al 1987), although free radicals may be important in intracellular killing. In the light of the extensive lipid peroxidation seen in MHV-3 infected livers, and the ability to abrogate the liver necrosis with certain free radical inhibitors, it is unlikely that NK cells are the primary cell involved.

Role of antibody and complement The role of antibody in this model has not been defined; however, there does not appear to be any neutralizing antibody present, as even a few PFU's (eg 1-10) of virus can induce a fulminant necrosis. Non-neutralizing antibody may play a much greater role. Our demonstration of the apparent presence of cross-reactive antibody to MHV-3 by RIA underlines the neccessity of using a panel of tests to determine positivity, as pointed out by Talbot and Buchmeir (1984), who found differing degrees of sensitivity and positivity among their assays. They felt this was because the amount of antibody present may be of borderline detectability, especially in very young mice.

Other workers (Fujiwara 1971, Talbot et al 1985, Lussier 1986) have clearly demonstrated the ubiquity of sub-clinical MHV infections in mouse colonies in Canada and the USA. There is therefore little doubt that these mice, in light of our confirmatory

antibody studies (RIA), have previously been exposed to MHV, and carry anti-MHV antibodies. The parasinusoidal deposits of IgG seen in both pre and recently infected mice, the latter well before a primary antibody response could be effected, also suggest prior antigen exposure. Infection with MHV-3 may, in this case, induce a secondary immune reponse. The formation of antibody complexes between viral antigen and non-neutralizing antibody has been known to enhance FcR mediated uptake of viruses by macrophages (Casali and Oldstone 1980). In a rat model where brain macrophages were infected with MHV-4, addition of purified rabbit IgG to the cell cultures increased the number of cells initially infected tenfold (Massa et al 1986). It is possible that the presence of IgG due to a previous subclinical infection with another MHV strain (i.e. MHV-S) may be partly responsible for the overwhelming nature of the reponse in Balbc/J mice, resulting in fulminant hepatitis.

It is important to note here that the susceptible Balbc/J mice have an intact complement system. C5 is important in both macrophage chemotaxis and phagocytosis. A deficiency of C5 would directly decrease C5a (a potent chemotactic fragment for both PMN's and monocyte/macrophages) and C5 participation in formation of the membrane attack complex. A deficiency of C5 would also be expected to result in decreased antibody mediated cytotoxicity, decreased local permeability after antigen challenge, decreased platelet aggregation, and decreased respiratory burst due to immunoglobulin coated particles (Wetsel et al 1987, McCarty & Synderman 1986, Rosenberg and Tachibana 1986). The presence of IgG plus an intact complement cascade in the susceptible mice is significant because these two factors have a synergistic effect on phagocytosis by macrophages (Unkeles and Wright 1988). Both viruses and aggregates of immunoglobulin activate the alternative complement pathway, while viruses and specific antibody can activate the classical pathway (Muller-Eberhard 1988). C5a also has profound effects on the microvasculature, causing platelet aggregation and increased

vascular permeability (Hugli et al 1987). The presence of C5a in the Balbc/J mice may augment antigen specific and non-specific antibody responses, possibly by stimulating IL-1 release from macrophages (Goldstein 88). C5a also stimulates release of PCA (tissue factor) from macrophages, (which is known to be elevated in this strain post infection), thus activating the coagulation cascade. Furthermore, depletion of complement can prevent oxyradical mediated damage from activated PMN's in an acute lung injury model (Till and Ward 1986).

Small immune complexes are frequently deposited on endothelial surfaces with consequent complement dependent injury to vessel walls. Immune complexes are also themselves stimulators of both the oxidative burst with free radical release and of the cyclooxygenase pathway of arachidonic acid metabolism (Morley et al 1979) with resultant release of thromboxane B2 (TxB2), a potent platelet aggregator. Dengue virus (DV) infection, a Flavovirus infection of humans, is associated with a syndrome, Dengue Hemorrhagic Fever (DHF), which has recently been shown to result from a second infection with a different strain of dengue virus. A second related infection induces an immune complex-mediated shock syndrome with disseminated intravascular coagulation (DIC) (Halstead et al 1984, Alvarez and Ramirez-Ronda 1985) which is accompanied by a macrophage-mediated T-cell suppression (Shukla and Chaturvedi 1984, Wong et al 1984). The presence of nonneutralizing cross-reactive antibody to MHV-3 may induce a similar response to that seen after a second dengue infection. That is, the presence of cross reactive antibody to MHV-3 may enhance uptake of virus, replication in the macrophages, and induce a DIC -like response in the susceptible Balbc/J mice, with amplified parenchymal necrosis due to secondary localized ischemic injury.

A/J mice may be protected from injury by their relative deficiency in C5 (Rosenberg and Tachibana 1986). This may prevent the pathogenic sequence of immune complex (MHV-

3 plus preformed cross reactive antibody) and complement activation of Kupffer cells (and/or endothelial cells and other macrophages) via the FcR, with subsequent release of reactive oxygen species, procoagulant activity, and platelet activating factor, leading to activation of coagulation and possible formation of microthrombi, and subsequent damage to hepatocytes.

Free Radical Reaction Products: Cause of Injury or Result of MHV-3 Infection?
There is an increasing body of experimental evidence indicating that toxic oxygen products generated by activated monocytes/macrophages are potent mediators of tissue injury. Partially reduced products of oxygen such as O_2^- and HO$\cdot$ contain unpaired electrons which will readily interact with the nearest available hydrogen, removing it from the lipid or protein. This can initiate a chain reaction, as HO$\cdot$ acts as a H atom extractor. The reaction is propagated when the molecule that is now missing a hydrogen, itself functions as a hydrogen extractor, and replaces it's missing hydrogen from a nearby molecule. The reaction is terminated when a molecule with an extra hydrogen interacts with a molecule with a missing hydrogen. In addition, some of the endproducts of oxidative cleavage of polyunsaturated fatty acids (alkenols, malondialdehyde) also have toxic effects, and induce methylation or strand scission in DNA.

Monocyte/macrophages and PMN's normally produce oxyradicals in response to foreign particles, and this is the main mechanism of dealing with bacterial infections. Any stimulators of phagocytosis, even if the stimulating agent is not internalized, activate the oxidative respiratory pathways in monocytes with subsequent extra-cellular release of free radicals. The cellular location of free radical production is the plasma membrane and plasmalemma surfaces, as well as the mitochondria (Goldstein et al 1977). Plasma membranes are critical sites for free radical injury but radical self-injury is normally

controlled by endogenous cytosolic and mitochondrial anti-oxidants such as catalase (CAT), superoxide dismutase (SOD), glutathione and Vitamin E. Extracellular fluids have a much lower concentration of scavengers and therefore, when monocytes are involved in microbiocidal phagocytosis, such extracellular release of FR's are important in augmentation of the inflammatory response (due to local injury and release of chemoattractants) with resultant control of the infectious agent. When the stimulus is ongoing (i.e. in the case of an immune complex disease) the continued activation of the inflammatory response may be responsible for the pathogenesis of the injury rather than being a positive response. The role of free radicals in tissue injury has been extensively reviewed (DelMaestro et al 1980, Freeman and Crapo 1982, Ward 1983, Aust and White 1985, Ward et al 1987, Gutteridge 1985,1988).

If the rate of extracellular release of free radicals exceeds the scavenging capacity of extracellular antioxidants, the excess superoxide and H_2O_2 may lead to generation of the highly toxic OH^- radical in the presence of metal catalysts. When OH^- reacts with a lipid on a nearby membrane, extracting a hydrogen, it initiates a chain-reaction with resultant lipid peroxidation and formation of aldehydes (such as malonaldelyde) and conjugated dienes. When malonaldehyde reacts with amine groups it yields certain fluorescent products (Freeman and Crapo 1982). Malonaldehyde is also known to induce damage via reaction with nitrogen containing bases in DNA.

In these studies we have assessed the possibility of oxyradical mediated injury by looking for radical-induced changes in lipids and proteins in the virally-infected liver. Three out of four of the tests chosen showed increased reaction products at 12 hours p.i. in the susceptible strain mice and no increase in the resistant A/J strain. This indicates that there has been excess production of radicals, i.e. more than can be inactivated by the available

supply of endogenous scavengers (such as glutathione, Vitamin E, superoxide dismutase).

Implications of the Inhibitor Studies Because of requirement for metal ions in in vivo lipid peroxidation (Aust and White 1985) deferoxamine, an iron-chelating agent, was used to inhibit this reaction. Deferoxamine plus nicotinamide treatment has been recently used in a pancreatic islet allograft model of Type I diabetes. Non-obese diabetic (NOD) mice were used as recipients, and Balbc/J mice as donors of pancreatic islet cells. Deferoxamine or nicotinamide alone, delayed destruction of islet grafts, while the combined treatment was effective in protecting islet tissue in 6/7 animals (Nomikos et al 1986). Compound U74006F has been shown to be a powerful inhibitor of iron-dependent lipid peroxidation. At a concentration of 10^{-4} M, deferoxamine, alpha-tocopherol, BHT, and U74006F all inhibited lipid peroxidation to a similar (90-100%) extent in a rat brain homogenate system of Fe2+ induced lipid peroxidation (Braughler et al 1987). In the in vivo scavenger experiments performed in this study, both U74006F and deferoxamine treatment (without MHV-3 infection) lowered the base level of conjugated dienes found in the untreated control mice by a mean of 13-14%. Deferoxamine plus nicotinamide did tend to decrease conjugated diene formation and significantly reduced necrosis. It is possible that even though the doses of these compounds employed were effective in other experimental forms of tissue injury, they were either too low to fully prevent the fulminant damage in this model, or were unable to fully reach the site of action. Lazeroid U74006F, a 21-amino steroid (a synthetic iron chelator developed by Upjohn Co.) in the 1 mg/kg/bid dose was more effective in reducing conjugated diene formation (from 79% to 14%) than 10 or 100 mg/kg/bid doses. However, increasing doses of Lazeroid did decrease liver necrosis (Table 5). This shows that conjugated diene formation and liver necrosis are not necessarily linked

proportionally. This also suggests that Lazeroid itself may have the ability to induce conjugated diene formation, when given in high doses.

Two further inhibitors (Table 6), superoxide dismutase plus catalase and N,N1-diphenyl-p-phenylenediamine (DPPD) were tested in this model. The combination of superoxide dismutase and catalase had no effect on liver necrosis or on conjugated diene formation. Possibly, the short half-life of these compounds renders them ineffective in a model where the release of free radicals, as from macrophages, may be sustained. If the site of initial attack by radicals is indeed the sinusoidal lining cells underlying the Kupffer cells, there may also be a problem of access by the inhibitors. DPPD is a well known inhibitor of lipid peroxidation. DPPD had a significant effect on liver necrosis but a lesser effect on conjugated diene formation. This discrepancy between the effect of DPPD on liver cell necrosis and lipid peroxidation was also seen in a CCL_4 model of hepatotoxicity (Torrielli et al 1974). In this study it became apparent that DPPD not only had antioxidant properties but also had an effect on microsomal enzyme function. Recent evidence (Quanguan and Moldeus 1988) regarding the mechanism of action of DPPD shows that it can powerfully inhibit metabolic processes dependent on cytochrome P-450.

Role of PAF, PCA & other Macrophage Products: PAF (1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine or AGEPC or PAF-acether) was first isolated from rabbit basophils (Terashita et al 1985), and is now known to be produced by a variety of activated cells: PMN's, endothelial cells, monocytes and macrophages, eosinophils, among other cells (Pinckard et al 88). PAF has numerous functions in vivo in addition to inducing degranulation and aggregation of platelets. Endothelial cells in the presence of thrombin produce PAF, and hence may induce PMN degranulation and hence amplify endothelial injury.

In a hamster cheek pouch model, extravascular application of PAF induced massive fluid extravasation, stimulated leukocyte adhesion and migration, and increased release of leukotrienes C_4 and D_4. PAF also induced PMN degranulation, arachidonic acid metabolism and oxygen radical production (Bjork and Smedegird 1983).

PAF mediates platelet aggregation and release of platelet granules in some animal species (Robertson and Smith 1986). PAF also acts in synergy with certain lipooxygenase and cyclooxygenase products as well as with thrombin and aggregated IgG (Pinckard et al 1988). Although high concentrations of PAF are required when used alone to induce superoxide production by PMN's, in synergy with other autocoids, low concentrations of PAF appear to prime for superoxide release.

A specific PAF antagonist CV3988 has been used to block activation of neutrophils and subsequent hydroxyl radical release (Barefoot et al 1985), to inhibit the inflammatory response to injected PAF (Issekutz and Szpejda 1986), to inhibit platelet aggregation (Robertson and Smith 1986), and to attenuate hypotension due to PAF and endotoxemia (Toth and Mikulaschek 1986). This may explain the inability of the combination of superoxide dismutase and catalase to have a significant effect on necrosis and conjugated diene production in this model. In the combination studies (Table 7), CV3988, a platelet activating factor (PAF) antagonist, was chosen to complement the free radical inhibitors. Previous work in this model (Levy et al 1983, MacPhee et al 1985) had demonstrated the release of monocyte procoagulant activity, and increase in plasma fibrinogen in the susceptible Balbc/J strain of mice post-infection. In addition, in-vivo studies suggested the formation of microthrombi early in the course of the disease. Furthermore, Ancrod, a defibrinating agent abrogated the course of the disease. It seemed important then to test the combined effects of intervention in both free radical injury and in activation of coagulation. The combination of CV3988 with a dose of superoxide dismutase plus

catalase that was ineffective by itself, had a significant effect on both conjugated diene levels and on liver necrosis. Similarily, when CV3988 was combined with the iron chelator DPPD both conjugated dienes and necrosis were reduced. The synergistic effect of such inhibitors of both free radical injury and activation of coagulation suggests that more that one macrophage activation product is important is the onset of injury to hepatocytes. It is quite possible that hepatocellular necrosis may be a result of the interaction of numerous (if not all) of the products released by activated macrophages.

CONCLUSIONS

In the course of these studies a number of significant new observations have been made:
1. Microvascular injury occuring in vivo has been documented, along with the movement of certain liver cells upstream. In vivo microcirculatory studies have shown that a decrease in red blood cell velocity in the hepatic sinusoids is one of the earliest functional changes after MHV-3 infection. These changes precede any local blockages of sinusoids and are associated with edema of one or several hepatocytes.

The movement of cells upstream has numerous and far-reaching implications regarding normal directed cell movement in general, and for all types of cell migration whether normal, inflammatory, or in response to a stimulus of any kind. It is now possible to envisage a cell receiving information from upstream and moving towards it without postulating cell detachment and complex routes of carriage by the blood stream until the cell reaches the upstream postition. Although we have been able to estimate migration rates of individual cells - much work remains to be done in determing "normal" stimulated and maximum cell movement rates.

2. It has been established that hepatocellular injury after MHV-3 infection is not T-cell mediated. Immunofluorescent cell surface marker studies of the cellular infiltrate, in vitro lymphocyte proliferation data and the lack of effect of cyclosporine A on developement of the hepatocellular necrosis all indicate that T-cells are neither present nor active in the tissue injury.

3. Mononuclear phagocytes play a principle role. Electron microscopic data supports the hypothesis of hepatocyte injury due to release of excited oxygen radicals and other products from Kupffer cells. Scanning electron microscopy of corrosion casts of MHV-3 infected livers have shown lesions remarkably uniform in size and shape. This indicates a point source of initiation of injury ,as well as a uniform time of initiation. This is also consistant with Kupffer cells as the initiating focus.

a) Strain dependent differences exist in macrophage chemiluminescence in response to MHV-3 infection. Resistant A/J strain mouse macrophages do not respond to opsonized zymosan stimulation with enhanced chemiluminesce , whereas the susceptible Balbc/J mice show a marked increase in chemiluminescence.

b). Strain dependent differences exist in mononuclear cell response after exposure to MHV-3 . Splenic mononuclear cells from the resistant A/J mice had a four fold greater proliferation response to MHV-3 than did Balbc/J mice. The crossed -culture studies determined that the difference lay with the MHV-3 antigen presenting cells and not with the responding lymphocytes. Observations a and b taken together suggest that substantial differences exist between macrophages from susceptible and resistant mouse strains, differences which may affect the immune response to the virus in vivo.

4. The presence of both free radical reaction products in MHV-3 infected livers and the ability of known free radical inhibitors to decrease liver necrosis has been

demonstrated. This suggests that toxic oxygen species play a significant role in the development of hepatocellular necrosis after infection with MHV-3.

5. Other macrophage activation products associated with injury. A platelet-activiting factor antagonist was found to have a synergistic effect when combined with the free radical scavengers superoxide dismutase and catalase. Our previous work in this model implicated monocyte procoagulant activity in susceptiblity , and plasminogen activator in resistance to MHV-3. Together this is very strong evidence that macrophage activation products are of primary importance in the pathogenesis of the hepatocellular necrosis.

6. Role of antibody to MHV-3. The presence of crossreactive antibody to MHV-3 is itself not unexpected (despite the mice being kept in microisolator units), due to the ubiquity of non-virulent MHV-S in mouse colonies. However, when combined with the other strain specific differences between A/J and Balbc/J mice (e.g. complement deficiency in A/J), it may be important in the induction of fulminant hepatic necrosis in the complement replete Balbc/J mice.

When this project began, the starting hypothesis was that T-cells may play a main role in the pathogenisis of the hepatocellular injury. However, the evidence strongly suggests that MHV induced cytotoxicity is not due to T-cell mediated events (see Fig 22 for flowchart of possible mechanism of injury). The sequence appears to consist of: uptake of virus by the Kupffer cells in the liver (possibly in the form of antigen-antibody complexes in a presensitized animal), followed by activation of, or injury to the Kupffer and/or endothelial cells, with extracellular release of free radicals and factors such as mPCA and PAF resulting in hepatocellular injury. A secondary possiblity is the uptake of virus by endothelial cells, with release of cytokines or presentation of antigen to the

Kupffer cells. The hepatocellular injury may result from lipid peroxidation by free radicals of hepatocellular plasma membranes adjacent to activated cells. The release of mPCA and PAF may initiate local clotting (which can be seen in-vivo) and hence compound hepatocellular injury via microenvironmental hypoxia. The simultaneous release of other macrophage products such as TNF (tumor necrosis factor) has not been excluded.

This mechanism could account for acute liver damage in other viral infections where the tissue injury is not clearly T-cell mediated. It may be that the protective effect of cyanidanol-3, recently reported to be of some benefit in clinical trials in chronic human hepatitis (Blum 1977, Piazza et al 1983 , Kakumu et al 1983, Suzuki et al 1986), is partially attributable to its free radical scavenging properties (Slater and Eakins 1975, Keeling et al 1986). Susceptibility to viral injury may ultimately depend on many interacting factors including the ability of the host to make neutralizing antibody, the presence of pre-formed antibody and complement which may be important in viral uptake, the extent of activation of the macrophage and release of toxic products and the presence or absence of sufficient extracellular free radical scavengers.

REFERENCES

Alper CA, MS Kruskall, D Marcus-Babley, DE Craven, AJ Katz, SJ Brink, JL Dienstag, Z Awdeh and EJ Yunis. (1989) Genetic prediction of nonresponse to hepatitis B vaccine. NEJM 321 (11):708-712.

Alvarez ME and CH Ramirez-Ronda. (1985) Dengue and hepatic failure. Am J Med 79:670-674.

Andzhaparidze AG, MS Balayan, AP Savinov, DM Braginskii, VF Poleshchuk and NA Zamyatina. (1986). Production in monkeys of a non A, non B hepatitis, transmitted by the fecal-oral route. Vop Virusol (1):73-80.

Aust SD, and BC White. (1985) Iron chelation prevents tissue injury following ischemia. Adv Free Rad Biol Med 1:1-17.

Babior BM. (1978) Oxygen-dependent microbial killing by phagocytes. NEJM 298 (12):659-668.

Bang FB, and A Warwick. (1960) Mouse macrophages as host cells for the hepatitis virus and the genetic basis of their susceptibility. PNAS 46: 1065-1075.

Balayan MS, AG Andjaparidze, SS Savinskaya, ES Ketiladze, DM Braginsky, AP Savinov and VF Poleschuk. (1983) Evidence of a virus in nonA, nonB hepatitis transmitted via the fecal-oral route. Intervirology 20:23-31.

Barefoot St, FW Kleinhans and PD Toth. (1985) Platelet activating factor (PAF) activation of neutrophils selectively blocked by a PAF analog. Fed Proc 44(4):1041.

Belabbes EH, A Bouguermouh, A Benatallah and G Illoul. (1985) Epidemic nonA, nonB viral hepatitis in Algeria: Strong evidence for its spreading by water. J Med Virol 16:257-263.

Bishop CJ, CM Rzepczyk, D Stenzel and K Anderson. (1987) The role of reactive oxygen metabolites in lymphocyte-mediated cytolysis. J Cell Sci 87:473-481.

Bjork J and G Smedegird. (1983) Acute microvascular effects of PAF-acether, as studied by intravital microscopy. Eur J Pharmacol 96:87-94.

Blum AH, W Soelle, K Kortum, P Peter and G Strohmeyer . (1977) Treatment of acute viral hepatitis with (+)-cyanidol-3. Lancet 2:1153.

Blumberg BS, BJS Gerstley, DA Hungerford, WT London and AI Sutnick. (1967) A serum antigen (Austrailia Antigen) in Down's syndrome, leukemia, and hepatitis. Ann Int Med 66(5):924-931.

Boss JH and WA Jones. (1963) Effect of certain murine pathogens on phagocytic activity. Br J Exp Path 46:433-422.

Bouwens L. (1988) Structural and functional aspects of kupffer cells Rev Sobre Biol Cell 16:69-94.

Bouwens L and E Wisse. (1989) Hepatic pit cells have natural cytotoxic (NC) activity against solid tumor -derived target cells. In: Cells of the hepatic sinusoid, Vol II. Wisse E, DL Knook and K Decker (eds), Kupffer cell foundation, Rijswijk, p 215-220.

Bouwens L, L Remels, Baekeland M, Van Bossuyt and E Wisse. (1987) Large granular lymphocytes or "Pit cells" from rat liver: isolation, ultrastructural characterization and natural killer activity. Eur J Immunol 17:37-42.

Bowens L, M Bakeland and E Wisse. (1986) A Balanced view of the origin of Kupffer cells. In: Cells of the hepatic sinusoid, Vol I. Kirn A, DL Knook and E Wisse (eds), Kupffer Cell Foundation, p 7-12.

Bowers GJ, TJ MacVittie, EF Hirsch, JC Conklin, RD Nelson, RJ Roethel and MP Fink. (1985) Prostanoid production by lipopolysaccharide-stimulated kupffer cells. J Surg Res: 501-508.

Bradley D, A Andjaparidze, EH Cook Jr., K McCaustland, M Balayan, H Stetter, O Velaquez, B Robertson, C Humphrey, M Knae and I Weisfuse. (1988) Aetiological agent of enterically transmitted nonA-nonB hepatitis. J Gen Virol 69:731-738.

Braughler JM, JF Pregenzer, RH Chase, LA Duncan, EJ Jacobsen and JM McCall. (1987) Novel 21-amino steriods as potent inhibitors of iron-dependent lipid peroxidation. J Biol Chem 262(22):10438-10440.

Brigelius R, C Muckel, TPM Akerboom and H Sies. (1983) Identification and quantification of glutathione in hepatic protein mixed disulfides and its relationship to glutathione disulfide. Biochem Pharmacol 32(17):2529-2534.

Brower A, AM DeLeeuw, DP Praaning-van Dalen and DL Knook. (1982) Isolation and culture of sinusoidal liver cells: Summary of a round table discussion. In: Sinusoidal Liver cells. Knook DL and E Wisse (eds), Elsevier Biomedical Press, p 509-516.

Burch GE, CY Tsui and JM Harb. (1973) Hepatitis in mice infected with Coxsackie virus B1. Br J Exp Pathol 54:249-254.

Casali P and MBA Oldstone. (1980) Immune complexes in viral infection. Adv Immunol 29:7-48.

Carr I . (1977) The Kupffer cell- an overview. In: Kupffer cells and other liver sinusoidal cells. Wisse E and DL Knook (eds), Elsevier/North-Holland Biomedical Press, Amsterdam, p 355-364.

Casteleijn E, J Kuiper, HC van Rooij, JA Kampus, JF Koster and TJ van Berkel. (1988) Hormonal control of glycogenolysis in parenchymal liver cells by Kupffer and endothelial liver cells. J Biol Chem 263(6):2699-703.

Cliff WJ. (1966). The behavior of macrophage labelled with colloidal carbon during wound healing in rabbit ear chambers. Quart J Exp Physiol 51:112-119.

Decker T, ML Lohmann-Matthes, U Karck, T Peters and K Decker. (1989) Comparative study of cytotoxicity, tumor necrosis factor, and prostaglandin release after stimulation of rat Kupffer cells, murine Kupffer cells, and murine inflammatory liver macrophages. J Leuk Biol 45(2):139-46.

Decker T, Kiderlen AF and ML Lohmann-Matthes. (1985) Liver macrophages (Kupffer cells) as cytotoxic effector cells in extracellular and intracellular cytotoxicity. J Infect Immunol 50(2):358-364.

DelMaestro RF, HH Thaw, J Bjork, M Planker and KE Arfors. (1980) Free radicals as mediators of tissue injury. J Physiol Scand Suppl 492:43-57.

Dick GWA, JSF Niven and AW Gledhill. (1955) A virus related to that causing hepatitis in mice (MHV). Br J Exp Path 36:90-98.

Diesselhoff-Den-Dulk MMC, RW Crofton and R Van Furth. (1979) Origin and kinetics of Kupffer cells during an acute inflammatory response. Immunology 37:7-14.

Dieter P, A Schulze-Specking, U Karck and K Decker. (1987) Prostaglandin release but not superoxide production by rat Kupffer cells stimulated *in vitro* depends on Na+/H+ exchange. Eur J Biochem 170(1-2):201-206.

Dieter P, A Schulze-Specking, S Eyhorn and K Decker. (1986) Glucocorticoids inhibit prostaglandin formation by rat kupffer cells primary culture. In: Cells of the Hepatic Sinusoid, Vol I, Kirn A, DL Knook and E Wisse (eds), Kupffer Cell Foundation, p 77-82.

Dindzans VJ, PJ MacPhee, LS Fung, JL Leibowitz and GA Levy. (1985) The immune response to mouse hepatitis virus: expression of monocyte procoagulant activity and plasminogen activator during infection *in vivo*. J Immunol 135(6):4189-4197.

Dixon JE, JE Allan, PC Doherty and Da Hume. (1986) Immunohistochemical analysis of the involvement of F4/80 and Ia-positive macrophages in mouse liver infected with lymphocytic choriomeningitis virus. J Leuk Biol 40(5):617-28.

Enzan H, S Iwamura, S Watanabe, T Saibara, S Ohnishi, Y Yamamoto, H Yamamoto and H Itara. (1989) Immunoelectron microscopic identification of pit cells in the rat and mouse livers. In: Cells of the hepatic sinusoid, Vol II, Wisse E, DL Knook and K Decker (eds), Kupffer Cell Foundation, Rijswijk, p 479-480.

Fahimi HD. (1982) Sinusoidal endothelial cells and perisinusoidal fat-storing cells: structure and function. In: The Liver: Biology and pathobiology. Arias I, H Popper, D Schachterand and DA Shafritz (eds), Raven Press, NY, p 1495-1506.

Farber JL. (1982) Biology of disease: Membrane injury and calcium homeostasis in the pathogenesis of coagulative necrosis. Lab Invest 47:114-122.

Favorov Mo, PA Khulchlovich, GK Zairov, EK Listouskaya, SA Arakelov, AN Vzorov, VM Stakhanova, ES Ketiladzea and VM Zhdarov. (1986) Clinical and epidemiological features and diagnosis of viral non A,non B hepatitis with a fecal-oral transmission mechanism. Vop Virusol 1:65-69.

Feinstone SM, AZ Kapikian and RH Purcell. (1973) Hepatitis A: Detection by immune electron microscopy of a viruslike antigen associated with acute illness. Science 182:1026-1028.

Feurer C, LH Chow and JF Borel. (1988) Preventative and therapeutic effects of cyclosporine and valine 2 dihydro-cyclosporine in chronic relapsing experimental allergic encephalomyelitis in the Lewis rat. Immunology 63:219-223.

Filice GA. (1988) Antimicrobial properties of Kupffer cells. Infect Immun 56(6):1430-1435.

Freeman BA and JO Crapo. (1982) Biology of disease: Free radicals and tissue injury. Lab Invest 47(5):412-426.

Fujiwara K. (1971) Problems in checking inapparent infections in laboratory mouse colonies: An attempt at serological checking by anamestic response. In: Defining of the laboratory animal. Schneider HA (ed). National Academy of Science, Washington, D.C.

Fujiwara K, I Ogata and S Mochida. (1989) *In situ* evaluation of stimulatory state of hepatic macrophage based on ability to produce superoxide in rats. In: Cells of the hepatic sinusoid, Vol II, Wisse E, DL Knook and K Decker (eds), Rijswijk, p 204-205.

Geerts A, P Schellinck, L Bouwens and E Wisse. (1988) Cell population kinetics of Kupffer cells during the onset of fibrosis in rat liver by chronic carbon tetrachloride administration. J Hepatol 6(1):50-56.

Goldin H and Y Keisari. (1986) Increased oxidative burst potential exhibited by macrophages during graft vs host reactions in mice. Transplantation 41(6):755-758.

Goldstein IM. (1988) Complement: Biologically active products. In: Inflammation: Basic principles and clinical correlates. JI Gallin, IM Goldstein and R Snyderman (eds). Raven Press, Ltd, NY, p 55-74.

Goldstein IM, M Cerguerira, S Lind and H Kaplan. (1977) Evidence that the superoxide-generating septum of human leukocytes is associated with the cell surface. J Clin Invest 59:249-254.

Greaves MF. (1976) Virus receptors on Lymphocytes. Scand J Immunol 5:113-123.

Gupta H, YK Joshi and BN Tandon. (1988) An enyzme-linked immunoassay for the possible detection of non-A, non-B viral antigen in patients with epidemic viral hepatitis. Liver 8:111-115.

Gut JP, M Anton, A Bingen, JM Vetter, and A Kirn. (1981) Frog Virus 3 induces a fatal hepatitis in rats. Lab Invest 45:218-228.

Gutteridge JMC. (1987) Lipid peroxidation : Some problems and concepts. In: Upjohn Symposium on Oxygen Radicals 1987, p 9-19.

Gutteridge JMC. (1985) the involvement of iron, copper and oxygen radicals in human diseases. Life Chemistry Reports 3:232-243.

Gutteridge JMC and GJ Quinlan. (1983) Malondialdehyde formation from lipid peroxides in the thiobarbituric acid test: The role of lipid radicals , iron salts, and metal chelators.

Hagmann W, AM Steffan A Kirn and D Keppler. (1987) Leukotrienes as mediators in frog virus 3-induced hepatitis in rats. Hepatology 7(4)732-736.

Halstead S, CN Venkateshaer, MK Gentry and LK Larsen. (1984) Heterogeneity of infection enhancement of dengue 2 strains by monoclonal antibodies. J Immunol 132(3):1529-1532.

Hampton JC. (1958) An electron microscope of the hepatic uptake and excretion of submicroscopic particles injected into the blood stream and into the bile duct. Acta Anat 32:262-291.

Hardonk MJ, FWJ Dijhuis, J Grond, J Koudstaal and S Poppema. (1986) Migration of Kupffer cells. In: Cells of the hepatic sinusoid, Vol I, Kirn A, DL Knook and E Wisse (eds), p 1-6.

Hawkins HK, JLE Ericsson, P Biberfeld and BF Trump. (1972) Lysosome and phagosome stability in lethal cell injury. Am J Path 68(2):255-272.

He LF, D Alling, T Popkin, M Shapiro, HJ Alter and RH Purcell. (1987) Determining the size of nonA, nonB hepatitis virus by filtration. J Infect Dis 156 (4):636-640.

Hightower JA, DL Earnest, AC Martens, C Zurcher, A Brouwer, E Blauw, AM de Leeuw and A Hagenbeek. (1987) Effects of acute graft-vs-host disease on the liver of the brown Norway rat. Leuk Biol 42(2):128-143.

Hirano T and BH Ruebner. (1966) Studies on the mechanism of destruction of lymphoid tissue in murine hepatitis virus (MHV-3) Infection. Lab Invest 15(1):270-282.

Hirsch MS. (1984) Pathogenesis of Viral Infections. In: Antiviral Agents and Viral diseases of Man. 2nd Ed. GJ. Galasso Raven Press, NY, p 35-54.

Holmes KV (1985) Replication of Coronavirus. In: Virology. BN Fields (ed). Raven Press. NY p 1331-1343.

Hugli TE, F Morceau and C Lundberg. (1987) Effects of complement fragments on pulmonary and vascular smooth muscle. Am Rev Respir 135:459-513

Inoue O, H Itakura, K Toriyama, A Satah, R Furukawa, Y Kusumoto, N Ishi, T Koji and S Nagataki. (1987) Ultrastructural study lymphocytic interaction with hepatocytes and endothelial cells in acute nonA, nonB hepatitis. Acta Pathol Jap 37(2):207-212.

Issekutz AC and M Szpejda. (1986) Evidence that platelet activating factor may mediate some acute inflammatory responses. Studies with the platelet activating factor antagonist, CV3988. Lab Invest 54(3):275-281.

Jones EA and JA Summerfield. (1982) Kupffer Cells. In: The Liver: Biology and Pathobiology. Arias I, H Popper, D Schachter and DA Shafritz (eds), Raven Press, NY, p 507-523.

Jungi TW and E Peterhans. (1988) Change in the chemiluminescence reactivity pattern during in vitro differentiation of human monocytes to macrophages. Blut 56:213-220.

Kakumu S, H Murakami and J Kuriki. (1983) Activation of suppressor function of human peripheral blood T Cells by (+) cyanidanol-3 : Its application to chronic active liver diseases. Clin Exp Immunol 52:430-436.

Karayiannis P, LM Petrovic, M Fey, D Moore, M Enticott, MJ McGarraey, PJ Scheurer and HC Thomas. (1989) Studies of GB Hepatitis in Tamarins. Hepatology 9(2):156-192.

Karck U, T Peters and K Decker. (1988) The release of tumor necrosis factor from endotoxin-stimulated rat Kupffer cells is regulated by prostaglandin E2 and dexamethasone. J Hepatol 7:352-361.

Keeling PWN, L Viola, MG Anderson, JH Marigold, JCM Macartney, FJ Pardinas, IM Murray-Lyon and RP Thompson. (1986) Trial of (+)-cyanidanol -3 in patients with Hepatitis B chronic Liver Disease. JR Soc Med 79(8):460-461.

Kingdon HS. (1987) Agents causing nonA, nonB hepatitis: could one be a prion? Med Hypoth 22:329-333.

Kirn A, Pierre, Bingen and A Steffan. (1983) Murine hepatitis induced by frog virus 3: A model for studying the effect of sinusoidal cell damage on the liver. Hepatology 3(1):105-111.

Kirn A, F Koehren and AM Steffan. (1982a) Interferon synthesis in primary culture of Kupffer and endothelial cells from the rat liver. Hepatology 2(5):676.

Kirn A, JP Gut and JL Gendrault. (1982b) Interaction of viruses with sinusoidal cells. In: Progress in Liver Diseases. Popper H and P Schaffner (eds), p 377-392.

Klatskin G. (1969) Hepatitis associated with systemic infections In: Diseases of the liver. L. Schiff (ed). J.B. Lippincott Company, Philadelphia, p 602-644.

Klein J. (1986) Natural history of the major histocompatibility complex. Wiley and Sons Publications, N.Y. p 309-368.

Koff RS and JT Galambos. (1987) Viral Hepatitis. In: Diseases of the liver. 6th Ed. Schiff L and ER Schiff(eds), JB Lippincott Company, Philadelphia, p 457-581.

Krauczynski K and DW Bradley. (1989) Enterically transmitted nonA, nonB hepatitis: Identification of virus associated antigen in experimentally infected Cynomalogous macaques. J Infect Dis 159(6):1042-1049.

Kuiper J, FJ Zijlstra, JA Kampus and TJ van Berkel. (1988a) Identification of prostaglandin D_2 as the major eicosanoid from liver endothelial and Kupffer cells. Biochem Biophys Acta 959(2):143-152.

Kuiper J, YB De Rijke, FJ Zijlstra, MP Van Waas, and TJ Van Berkel. (1988b) The induction of glycogenolysis in the perfused liver by platelet activating factor is mediated by prostaglandin D_2 from Kupffer cells. Biochem Biophys Res Comm 157:1288-1295.

Kurokawa S, H Ishibashi, M Shirahama, K Hayashida, Y Tsuchiya, Y Sakaki and Y Niho. (1988) Production of hepatocyte stimulating factor of rat Kupffer cells induced by lipopolysaccharide: Partial characterization and effects on alpha 2 macroglobulin gene expression in cultured adult rat hepatocytes. J Clin Lab Immunol 25(3):131-137.

Latham PS. (1988) The role of hepatocytes and sinusoidal cells in the pathogenesis of viral hepatitis. Int Rev Cytology 112:185-223.

LeRay D, C Dupuy and JM Dupuy. (1982) Immunopathology of mouse hepatitis virus type 3 infection. IV. MHV-3 induced immunodepression. Clin Immunol Immunopath 23:539-547.

Lever AML . (1987) Mechanisms of virally induced liver damage. J Hepatol 4:399-403.

Levy GA, PJ MacPhee, LS Fung, MM Fisher and AM Rappaport. (1983) The effect of mouse hepatitis virus infection on the microcirculation of the liver. Hepatology 3(6):964-973.

Levy GA, JL Leibowitz and TS Edgington. (1981) Induction of monocyte procoagulant activity by murine hepatitis virus type 3 (MHV-3) parallels disease susceptibility in mice. J Exp Med 154:1150-1163.

Lewin R. (1986) New class of animal virus found in virulent form of human hepatitis. Science 234(4775):423-424.

Lussier G. (1986) Prevalence of natural virus infections in laboratory mice and rats used in Canada. Lab An Sci 36(2):145-148.

MacDonald IC, DM Ragan, EE Schmidt, and AC Groom. (1987) Kinetics of red blood cell passage through interendothelial slits into venous sinuses in rat spleen, analyzed by *in vivo* microscopy. Microvasc Res 33:118-134.

MacNaughton MR and S Patterson. (1980) Mouse hepatitis virus strain 3 infection of C57, alsn and A/J strain mice and their macrophages. Arch Virol 66:71-77.

MacPhee PJ and PA Keown. (1989) Mechanisms of hepatic injury in viral (MHV-3) hepatitis: Role of macrophage production of excited oxygen radicals. In: Cells of the hepatic sinusoid. Vol II. Wisse E, Knock KL and D Decker (eds), p 363-4.

MacPhee PJ, EE Schmidt, PA Keown and AC Groom. (1988) Microcirculatory changes in livers of mice infected with murine hepatitis virus. Evidence from microcorrosion casts and measurements of red cell velocity. Microvasc Res. 36:140-149.

MacPhee PJ and GA Levy. (1985) The effect of a specific defibrinating agent (Ancrod) on mouse hepatitis virus (MHV-3) infection. Fed Proc 44(6):1840.

MacPhee PJ, VJ Dindzans, LS Fung, and GA Levy. (1985) Acute and chronic changes in the microcirculation of the liver in inbred strains of mice following infection with mouse hepatitis virus type 3. Hepatology 5(4):649-660.

Magilavy DB and JL Rothstein. (1988) Spontaneous production of tumor necrosis factor alpha by Kupffer cells of MRL/lpr mice. J Exp Med 168(2):789-794.

Maier RV and GS Hahnel. (1984) Microthrombosis during endotoxemia: Potential role of hepatic versus alveolar macrophages. J Surg Res 36:362-370.

Malter M, E Friedrich and R Suss. (1986) Liver as a tumor cell killing organ: Kupffer cells and natural killers. J Cancer Res 46(6):3055-3060.

Marshall JC, C Lee, JL Meakins, RP Michel and NV Christou. (1987) Kupffer cell modulation of the systemic immune response. Arch Surg 122(2):191-196.

Massa Pt, H Wege and V Ter Meulen. (1986) Analysis of murine hepatitis virus (JHMV strain) tropism toward Lewis rat glial cells *in vitro*. Type I astrocytes and brain macrophages (microglia) as primary glial cell targets. Lab Invest 55(3):318-327.

McCarty GA and R Snyderman. (1986) Component deficiencies. The fifth component. In: Hereditary and acquired complement deficencies in animals and man. Rother K and U Rother (eds). Prog Allergy 39:271-282.

McCuskey RS, PA McCuskey, R Urbaschek and B Urbaschek. (1987) Kupffer cell function in host defense. Rev Infect Dis. 5:S616-619.

McCuskey RS, PA McCuskey, JL Gendreault, B Ditter, K Becker, AM Steffan, and A Kirn. (1986) *In vivo* and electron microscopic study of dynamic events occuring in hepatic sinusoids following frog virus 3 infection. In: Cells of the hepatic sinusoids, Vol I, Kirn A, DL Knook and E Wisse, (eds). Kupffer cell foundation, p 351-356.

Metzger Z, JT Hoffeld, and JJ Oppenheim. (1980) Macrophage-mediated suppression. I Evidence for participation of both hydrogen peroxide and prostaglandins in suppression of murine lymphocyte proliferation. J Immunol 124(2):983-988.

Morahan PS, PH Coleman, SS Morse and A Volkman. (1982) Resistance to infections in mice with defects in the activities of mononuclear phagocytes and natural killer cells: Effects of immunomodulators in Beige mice and [89]Sr- treated mice. Infect Immun 37(3):1079-1085.

Morley J, MA Bray, RW Jones, DH Nugterenand and DA Van Dorp. (1979) Prostaglandin and thromboxane production by human and guinea-pig macrophages and leucocytes. Prostaglandins. 17:729-736.

Mosley JW and JT Galambos. (1969) Viral Hepatitis. In: Diseases of the Liver. L Schiff (ed), JB Lippincott, Philadelphia, p 410-497.

Muller-Eberhard HJ. (1988) Complement: Chemistry and pathways. In: Inflammation: Basic principles and clinical correlates. JI Gallin, IM Goldstein and R Syderman (eds), Raven Press Ltd, NY, p 21-53.

Muro H, H Shirasawa, Y Takahashi, M Maeda and S Nakamura. (1988) Localization of Fc receptors on liver sinusoidal endothelium. A histological study by electron microscopy. Acta Pathol Japonica. 38(3):291-301.

Muro H, H Shirasawa, M Maeda and S Nakamura. (1987) Fc receptors of liver sinusoidal endothelium in normal rats and humans. A histologic study with soluble immune complexes. Gastroenterology 93(5):1078-1085.

Murray HW and CF Nathan. (1988) *In vivo* killing of intracellular visceral leishmania donovani by a macrophage-targeted hydrogen peroxide-generating system. J Infect Dis 158(6):1372-1375.

Naito M, F Yamamura, M Takeya and K Takahashi . (1986) Ultrastructural analysis of kupffer cell progenitors. In: Cells of the hepatic sinusoid, Vol I . Kirn A, DL Knook and E Wisse (eds), Kupffer Cell Foundation, p 13-20.

Neiman JC, MJ Mant and TK Shnitka. (1987) Phagocytosis of platelets by Kupffer cells in immune thrombocytopenia. Arch Path Lab Med 111(6):563-565.

Nomikos IN, SJ Prowse, P Carotenuto and KJ Lafferty. (1986) Combined treatment with nicotinamide and desferrioxamine prevents islet allograft destruction in NOD mice. Diabetes 35:1302-1304.

Nopanitaya W, JG Aghajanian and LD Gray. (1979). An improved plastic mixture for corrosion casting of the gastrointestinal system. In: Scanning electron microscopy/III, SEM Inc. O'Hare, Illinois, p. 751-755.

Ouwendijk RJ, F Jzillstra, AM Van den Broik, A Brouwer, JH Wilson and JE Vincent. (1988) Comparison of the production of eicosanoids by human and rat peritoneal macrophages and rat Kupffer cells. Prostaglandins 35(3):437-466.

Parry EW. (1978) Studies on mobilization of Kupffer cells in mice. I The effect of carbon tetrachloride-induced liver necrosis. J Comp Path 88:481-495.

Paul P, SA Rothmann, JT McMahan and AS Gordon. (1984) Erythropoietin secretion by isolated rat kupffer cells. Exp Hematol 12:825-830.

Pereira CA, A Steffen and A Kirn. (1984) Kupffer and endothelial liver cell damage renders A/J mice susceptible to mouse hepatitis virus type 3. Virus Research 1:557-563.

Peterhans E, TW Jungi and R Stocker. (1988) Autotoxicity and reactive oxygen in viral disease. In: Oxy-radicals in molecular biology and pathology. Alan R Liss Inc., p. 543-562.

Peterhans E, T Baechi and J Yewdeil. (1983) Evidence for different receptor sites in mouse spleen cells for the Sendai virus hemagglutinin-neuraminidase (HN) and fusion (f) glycoproteins. Virol 128:366-376.

Peterhans E. (1980) Chemiluminescence: An early event in the interaction of Sendai and influenza viruses with mouse spleen cells. Virology 105:445-455.

Peterhans E. (1979) Sendei virus stimulates chemiluminescence in mouse spleen cells. Biochem Biophys Res Comm 91(1):383-392.

Petranyi GC, R Kiessling and G Zlein. (1975) Genetic control of natural killer lymphocytes in the mouse. Immunogenetics 2:53-61.

Phillips MJ, S Poucell, J Patterson and P Valencia. (1987a) The Normal Liver. In: The liver: An atlas and text of ultrastructural pathology. Raven Press, NY, p 1-36.

Phillips MJ, S Poucells, Patterson J and Valencia P. (1987b) Viral Hepatitis. In: The Liver: An atlas and text of ultrastructural pathology. Raven Press, NY, p 37-100.

Phillips MJ, Poucell S, Patterson J and Valencia P. (1987c) Drug and Toxic Effects. In: The Liver: An atlas and text of ultrastructural pathology. Raven Press, NY, p 159-238.

Phillips MJ, Poucell S, Patterson J and P Valencia. (1987d) Miscellaneous Hepatic Conditions. In: The Liver: An atlas and text of ultrastructural pathology. Raven Press, NY, p 519-567.

Piazza M, V Guadagnino, L Picciotto, R DeMercato, A Chirianni, R Orlando and G Golden. (1983) Effect of (+)- cyandidanol 3 in acute HAV, HBV, and non A, non B viral hepatitis. Hepatology 3(1):45-49.

Piguet PF, S Izui, A Janin-Mercier, Y Kapanci and P Vassalli. (1987) Interstitial pneumonitits and hepatitis after transfer of bone marrow cells bearing the lpr gene to

irradiated recipients: A disease due to large granular lymphocytes? Scand J Immunol 26:603-610.

Pinckard RN, JC Ludwig and LM McManus. (1988) Platelet-activating factors. In: Inflammation: Basic principles and clinical correlates. JI Gallin, JM Goldstein and R Snyderman, (eds), Raven Press Ltd, NY, p 139-167.

Prasad RN, RC Mahajan, KJ Virk and NK Ganguly. (1987) Enhanced complement synthesis by Kupffer cells during intestinal amoebiasis in guinea pigs. Ind J Med Res 86:181-184.

Provost PJ, BS Wolanski, WJ Miller, OL Ihensohn, WJ McAleer and MR Hilleman. (1975) Physical, chemical and morphological dimensions of human hepatitis A virus strain CR326 (385787). Proc Soc Exp Biol Med 148:532-539.

Quanguan J and P Moldeus. (1988) Effect of the antioxidant N, N-Diphenyl-p-Phenylenediamine (DPPD) on bromobenzene metabolism and toxicity in isolated hepatocytes. Pharm Toxicol 62:104-106.

Rappaport AM. (1976) The microcirculatory acinar concept of normal and pathological hepatic structures. Beitr Pathol Bd 157:215-243.

Recknagel RO, G Ugazio, RR Koch and EA Glende. (1973) New perspectives in the study of experimental carbon tetracholoride liver injury. In: The Liver. Gall EA and FK Mostofi (eds), Williams & Wilkins Co., Baltimore p 150-160.

Recknagel RO and EA Glende. (1973). Lipid peroxidation in acute carbon tetracholoride liver injury. In: Intermediary metabolism of the liver. Brown H and DR Hardwick (eds), CC Thomas Pubs., Springfield, Ill., p 23-57.

Reider H, G Ramadori, and KH Meyer zum Buschenfelde. (1988a) Guinea pig Kupffer cells can be activated *in vitro* to an enhanced superoxide response. I. Comparison with peritoneal macrophages. J Hepatol 7:338-344.

Reider H, G Ramadori, and KH Meyer zum Buschenfelde. (1988b) Guinea pig Kupffer cells can be activated *in vitro* to an enhanced superoxide response. II. Involvement of eicosanoids. J Hepatol 7:345-351.

Reider H, M Birmelin, and K Decker. (1982) Synthesis and functions of fibronectin in rat liver cells *in vitro*. In: Sinusidal Liver Cells, Knook DL and Wisse D (eds), Amsterdam, The Netherlands, Elsevier Press, p 193.

Richman LK, RJ Klingenstein, JA Richman, W Strober, and JA Berzofsky. (1979) The murine Kupffer cell. I Characterization of the cell serving acessory function in antigen-specific T-cell proliferation. J Immunol 123(6):2602-2609.

Rizzetto M, MG Canese, S Arico, O Crivelli, C Trepo, F Bonino and G Verme. (1977) Immunofluorescence detection of new antigen-antibody system (delta/anti-delta)

associated to hepatitis B virus in liver and in serum of HBsAg carriers. Gut 18(12):997-1003.

Robertson DN and GM Smith. (1986) CV3988 inhibits *in vivo* platelet aggregation induced by PAF acether and collagen. Eur J Pharmacol 123:91-97.

Rosenberg LT and DK Tachibana. (1986) Mice deficient in C5. Prog Allergy 39:169-191.

Rosner B. (1986) Hypothesis Testing: Two sample inference. In: Fundamentals of Biostatistics. PWS Publishers, Boston, p 240-267.

Ruebner BH, T Hirano and RJ Slusser. (1967) Electron microscopy of the hepatocellular and Kupffer-cell lesions of mouse hepatitis, with particular reference to the effect of cortisone. Am J Path 51(2):163-189.

Ruebner B and K Miyai. (1961) The Kupffer cell reaction in murine and human viral hepatitis with particular reference to the origin of acidophilic bodies. Am J Path 40:425-435.

Rush DN and PA Keown. (1984) Human monocyte chemiluminescence triggered by IgG aggregates. Requirement for phospholipase A activation and modulation by FcR ligands. Cell Immunol 87:252-258.

Sakagami Y, Y Mizoguchi, N Kawada, H Kuboi, S Seki, K Kobayashi, T Shin, H Takeda, K Kioka, and Y Ichikawa. (1988) Release of pepetide leukotrienes from rat Kupffer cells. Jap J Gastroenterol 85:1269-72.

Sancho J, E Gonzalez and J Egido. (1986) The importance of the Fc receptors for IgA in the recognition of IgA by mouse liver cells: Its comparison with carbohydrate and secretory component receptors. Immunology 57(1):37-42.

Sartwell PE. (1947) Infectious hepatitis in relation to blood transfusion. Bull US Army Med Dept 7:90-100.

Schubert WK, JC Partin and JS Partin. (1973) Encephalopathy and fatty liver (Reye's Syndrome). Prog Liver Dis p 489-510.

Schindler L, H Engler and H Kirchner. (1982) Activation of natural killer cells and induction of interferon after injection of mouse hepatitis virus type 3 in mice. Infect Immun 35(3):869-873.

Schmidt EE, IC MacDonald and AC Groom. (1990) Interactions of leukocytes with vessel walls and with other blood cells, studied by high resolution intravital video microscopy of spleen. Microvasc Res: In Press.

Sedlak J and RH Lindsay. (1968) Estimation of total protein bound and non-protein sulfhydral groups in tissue with Ellman's reagent. Anal Biochem 25:192-205.

Shirahama M, H Ishibashi, Y Tsuchiya, S Kurokawa, K Hayashida, Y Okumura, and Y Niho. (1988a) Kupffer cells may autoregulate interleukin 1 production by producing interleukin 1 inhibitor and prostaglandin E_2. Scand J Immunol 28:719-725.

Shirahama M, H Ishibashi, Y Tsuchiya, S Kurokawa, Y Okumura, and Y Niho. (1988b) Kinetics and parameters of the induction of interleukin 1 secretion by rat Kupffer cells. J Clin Lab Immunol 27:127-132.

Shukla MI and UC Chaturvedi. (1984) Study of the target cell of the dengue virus-induced suppressor signal. Br J Exp Path 65:267-273.

Slater TF and MN Eakins . (1975) Interactions of (+)-cyanidanol-3 with free radical operating systems. International Symposium Trends Therapy Liver Disease, Tirrenia 1974, Karger, Basel, p 84-89.

Sonderer B, P Wild, R Wyler, A Fontana, E Peterhans and M Schwyzer. (1987) Murine glia cells in culture can be stimulated to generate reactive oxygen. J Leuk Biol 42:463-473.

Steffan AM and A Kirn. (1986) C3-mediated phagocytosis induced in murine Kupffer cells by "in vitro" activation with endotoxin. Gastroenterol Clin Biol 10(2):117-121.

Steffan AM , GL Gendrault, RS McCuskey, PA McCuskey and A Kirn. (1986) Phagocytosis, an unrecognized property of murine endothelial liver cells. Hepatology 6(5):830-836.

Steffan AM and A Kirn. (1979) Multiplication of vaccinia virus in the livers of mice after frog virus 3 induced damage to sinusiodal cells. J RES 26(5):531-538.

Stohlman SA, PR Brayton, RC Harmon, D Stenesory, RG Ganges and GK Matsushima. (1983) Natural killer cell activity during mouse hepatitis virus infection: Response in the absence of interferon. Int J Cancer 31:309-314.

Stohr G, W Deimannn and HD Fahimi. (1978) Peroxidase positive endothelial cells in sinusoids of the mouse liver. J Histochem Cytochem 26(5):409-411.

Storkus WJ and JR Dawson. (1986) Oxygen-reactive metabolites are not detected at the effector-target interface during natural killing. J Leuk Biol 39:547-557.

Suzuki H, S Yamamoto, C Hirayama, T Takino, K Fujisawa and Y Oda. (1986) Cyanidanol therapy for HBe-antigen-positive chronic hepatitis:A multi-centre, double blind study. Liver 6(1):35-44.

Svoboda D, A Nielson, A Werder and J Higginson. (1962) An electron microscopic study of viral hepatitis in mice. Am J Path 41(2):205-224.

Taguchi F, S Kawamura and F Fujiwara. (1983) Replication of mouse hepatitis viruses with high and low virulence in cultured hepatocytes. Infect and Immun 39:955-959.

Talbot PJ, AA Salmi, RL Knobler and MJ Buchmeir. (1985) Epitope-specific antibody response to murine hepatitis virus -4 (strain JHM). J Immunol 134(2):1217-1224.

Talbot PJ, RL Knobler and MJ Buchmeier. (1984) Western and dot immunoblotting analysis of viral antigens and antibodies: Application to Murine Hepatitis Virus. J Immunol Meth 73:177-188.

Tanikawa K. (1979) Liver Diseases. In: Ultrastructural aspects of the liver and its disorders. 2nd Ed. Igaku-Shoin, Tokyo, p 174-238.

Terashita Z, Y Imura, K Nishikawa and S Sumida. (1985) Is platelet activating factor (PAF) a mediator of endotoxin shock? Eur J Pharmacol 109:257-261.

Till GO and PA Ward. (1986) Systemic complement activation and acute lung injury. Fed Proc 45(1):13-18.

Torrielli MV, G Ugazio, L Gabriel, and E Burdino. (1974) Time course of protection by N, N-Diphenyl-p-phenylendiamine (DPPD) against carbon tetrachloride hepatotoxicity. Agents Actions 4/5:383-390.

Toth PD and AW Mikulaschek. (1986) Effects of a platelet-activating factor antagonist, CV-3988, on different shock models in the rat. Circ Shock 20:193-203.

Trinchieri G and B Perussia. (1984) Biology of disease. Human natural killer cells: Biologic and pathologic aspects. Lab Invest 50(5):489-513.

Trump BF and IK Bejezesky. (1985) The role of calcium in cell injury and repair. Surv Synth Path Res 4:248-256.

Trump BF, PJ Goldblatt and RE Stowell. (1965) Studies of necrosis in vitro of mouse hepatic parenchymal cells. Lab Invest 14(11):1946-1968.

Unkeless JC and SD Wright. (1988) Phagocytic cells: Fc gamma and complement receptors. In: Inflammation: Basic principles and clinical correlates. JI Gallin, IM Goldstein and R Snyderman (eds), Raven Press Ltd, NY, p 343-362.

Van Kessel KPM, JAG Van Strijp, HJ Van Kats-Renaud, LAM Miltenburg, ME VanderTol, AC Fluit and J Verhoeff. (1987) Further evidence against a role for toxic oxygen products as lytic agents in NK cell-mediated cytotoxicity. Immunol 62:675-678.

Venkateswaran PS, I Millman and BS Blumberg. (1987) Effects of an extract from Phyllanthus niruri on heptitis B and woodchuck hepatitis viruses. In: In vitro and in vivo studies. PNAS USA 84:274-278.

Vincent PA, E Cho, and TM Saba. (1989) Effect of repetitive low-dose endotoxin on liver parenchumal and Kupffer cell fibronectin release. Hepatology 9:562-569.

Virk KJ, NK Ganguly, RC Mahajan, SR Bhushnurmath, and JB Dilawari. (1988) Generation of reactive oxygen species by Kupffer cells and blood monocytes during intestinal amebiasis in guinea pigs. Gastro Jpn 23:688-694.

Wake K. (1989) Rapid golgi method contributes notably to the study of liver sinusoidal cells. In: Cells of the hepatic sinusoid Vol II, Wisse E , DL Knook and K Decker (eds), p 449-450.

Ward PA. (1983) Role of toxic oxygen products from phagocytic cells in tissue injury. Adv Shock Res 10:27-34.

Ward PA, KJ Johnston, JS Warren and RG Kunkel. (1987) Immune complexes, oxygen radicals and lung injury. In: Upjohn Symposium on Oxygen Radicals, 1987, p 107-114.

Wardle EN. (1987) Kupffer cells and their function. Liver 7(2):63-75.

Warren JS, RG Kunkel, KJ Johnson and PA Ward. (1987) Comparative O_2-responses of lung macrophage and blood phagocytic cells in the rat. Possible relevance to IgA immune complex induced lung injury. Lab Invest 57(3):311-320.

Watanabe J, K Kanai and S Kanamura. (1988) Glucagon receptors in endothelial and Kupffer cells of mouse liver. J Histochem Cytochem 36(9):1081-1089.

Wege H, St Siddell and V TerMeulen. (1982) The biology and pathogenesis of coronaviruses. Curr Top Microbiol Immunol 99:131-200.

Werner-Wasik M, W von Muenchhausen, JP Nolan and SA Cohen. (1989) Endogeneous interferon alpha/beta produced by murine Kupffer cells augments liver-associated natural killing activity. Can Immunol Immunother 28(2):107-115.

Wetsel RA, RT Ogata and BF Tack. (1987) Primary structure of the fifth component of murine complement. Biochem 26:737-743.

Williams B, AM Prince, T Huima and B Botman. (1988) Spumaviruses isolated from sources containing agents of non A, non B (NANB) hepatitis do not cause NANB hepatitis. J Med Virol 24:263-274.

Wilson GA, S Beushausen and S Dales. (1986) In vitro models of demyelinating diseases. XV. Differentiation influences the regulation of coronavirus infection in primary explants of mouse CNS. Virology 151:253-264.

Wisse E, A Geerts, L Boumens, H VanBossuyt, K Vanderlerken and F Van Goetem. (1989) Cells of the hepatic sinusoid anno 1988. In: Cells of the hepatic sinusoid, Vol II, Wisse E, DL Knook and K Decker (eds), p 1-9.

Wisse E, JM Van Noordende, J Vander Meulen and WT Daems. (1976) The pit cell: description of a new type of cell occurring in the rat liver and peripheral blood. Cell Tissue Res 173:423-435.

Wong PY, S Deir, IFC McKenzie, KL Yap and T Pang. (1984) Induction and Ly phenotype of suppressor T-cells in mice during primary infection with dengue virus. Immunol 51:51-56.

Yokota S and K Kato. (1987) Immunocytochemical localization of cathepsins B and H in rat liver. Histochemistry 88(1):97-103.

Yam LT, CY Li and WH Crosby. (1971) Cytochemical identification of monocytes and granulocytes. Am J Clin Path 55:283-290.

Yamashita T, A Someya and E Hara. (1985) Response of superoxide anion production by guinea pig eosinophils to various soluble stimuli: Comparison to neutrophils. Arch Biochem Biophys 241(2):447-452.

Yoshida MC, R Masuda, M Sasaki, N Takeichi, H Kobayashi, K Dempo and M Mori (1987). New mutation causing hereditary hepatitis in the laboratory rat. J Hered 78:361-365.

Zairov GK, UM Stakhouva, EK Listovskaya, LA Konstantinova, MO Favorov, ES Ketiladze and M Zhdannov. (1986) Electron microscopic study of non A, non B viral hepatitis with a fecal-oral mechanism of transmission. Vop Virusol 2:172-175.

Zenilman ME, M Fiani, P Stahl, E Brunt and MW Flye. (1988) Use of ricin A-chain to selectively deplete Kupffer cells. J Surg Res 45(1):82-89.

Zerbe O and AM Gressner. (1988) Proliferation of fat-storing cells is stimulated by secretions of Kupffer cells from normal and injured liver. Exp Mol Pathol 49(1):87-101.

Zuckerman AJ. (1987) Hepatitis viruses. In: Principles and practises of clinical virology. Zuckerman AJ, JE Banatvala and JR Pattison (eds), John Wiley and Sons, NY, p 135-158.