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# The association between murine cytomegalovirus induced hepatitis and the accummulation of oval cells

HEIDI S. CASSELL*, PATRICIA PRICE†, STUART D. OLVER AND GEORGE C. T. YEOH*

**Department of Biochemistry, University of Western Australia, Nedlands, Western Australia, †Department of Clinical Immunology, Royal Perth Hospital, Perth, Australia and Queensland Institute of Medical Research, Herston, Queensland, Australia.*

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**Summary.** The accumulation of oval cells is an early event in the development of hepatocellular carcinoma induced by certain experimental regimes involving hepatocarcinogens. Oval cells have also been observed during chronic hepatitis induced by alcohol and iron overload. In this study, livers of murine cytomegalovirus (MCMV) infected mice were examined to determine whether hepatitis induced by this virus could initiate oval cell proliferation. BALB/c and C57BL/6 mice were infected with MCMV and studied 4, 8, 10 and 12 months later, alongside control (uninfected) mice. The livers were examined histochemically, immunocytochemically and by *in situ* hybridization to identify oval cells, inflammatory cells and proliferating cells. Oval cells were seen in the periportal regions of livers from MCMV infected BALB/c mice. These increased in number from 4 to 12 months after infection in parallel with increases in the numbers of inflammatory cells, even though cells expressing MCMV antigens were no longer evident in these samples. Proliferating oval cells and hepatocytes were identified by PCNA staining, indicating an increased level of liver regeneration in the infected livers. C57BL/6 mice are less susceptible to persistent MCMV hepatitis and had fewer oval cells than BALB/c mice. Thus the study demonstrates an association between MCMV induced hepatitis, inflammation, and presence of oval cells.

*Keywords:* oval cells, cytomegalovirus, hepatitis, carcinogenesis.

Several conditions which produce chronic liver damage predispose the host to hepatocellular carcinoma. These include excess alcohol (Yu *et al.* 1983), iron overload as a consequence of genetic haemochromatosis (Kew 1990) and hepatitis B infection (Hsia *et al.* 1992).

Correspondence: Dr Patricia Price, Department of Clinical Immunology, North Block level 2 – Royal Perth Hospital, Wellington Street, Perth 6000 Western Australia. Fax +61 8 9224 2920; E-mail: pprice@cyllene.uwa.edu.au

Cytomegaloviruses are a group of double stranded DNA viruses within the herpes family (Huang & Kowalik 1993). HCMV is endemic in all communities, with seropositivity rates of 50–100% in the third world. Most infections of immunocompetent individuals are subclinical, with a mononucleosis syndrome being the most common clinical manifestation in healthy adults and children. However, HCMV can induce severe hepatitis. Recent evidence suggests that all forms of HCMV disease affect the liver, and it is the most common cause of hepatitis in young children (Nigro *et al.* 1992). As yet, the

underlying mechanisms and the potential for HCMV to act as a cofactor for other forms of hepatic disease have not been addressed. This may include fibrosis, cirrhosis or hepatic carcinoma. For example; co-infection of hepatitis B patients with HCMV exacerbates disease attributed to hepatitis B, which directly or indirectly may increase the risk of hepatic carcinoma (Marinelli *et al*. 1993).

Oval cells are small epithelial like cells that have a scant pale cytoplasm, and relatively large ovoid nuclei. They are located periportally, more specifically, around the terminal ducts. Functionally, they have been assigned the role of a hepatic stem cell able to produce a differentiated progeny of the bile duct and hepatocyte lineages. Oval cells express oncodevelopmental proteins that are down regulated in mature hepatocytes (Tee *et al*. 1994). Hence they can be distinguished from other liver cells by their expression of foetal isoenzymes including the foetal form of pyruvate kinase (M2PK), and pi glutathione-S-transferase ($\pi$GST). Oval cells are present during the early stages of hepatocarcinogenesis induced by carcinogenic diets such as the choline deficient ethionine supplemented (CDE) diet (Shinozuka *et al*. 1978). The presence of oval cells during the early stages of hepatocarcinogenesis and their differentiation into hepatocytes, suggests that at least some hepatocellular carcinomas may be derived from oval cell progenitors (He *et al*. 1994; Thorgeirsson 1995). As the oncogenic potential of HCMV has been seen *in vitro* through the transformation of human fibroblasts into malignancy (Rosenthal & Choudhury 1993), cytomegaloviruses may be directly carcinogenic by promoting the accumulation of oval cells and/or by triggering oncogenic events in the replicating oval cell population.

We and others have previously shown that oval cells stain positively for albumin, transferrin, $\alpha$1-fetoprotein (AFP), pGST and M2PK (Sell 1983; Hayner *et al*. 1984; Tee *et al*. 1992; Smith *et al*. 1996; Tian *et al*. 1997). However, for identifying oval cells, albumin and transferrin are not useful markers because they are expressed in normal adult hepatocytes. AFP and $\pi$GST are expressed in some adult hepatocytes and bile duct cells in some liver pathologies (Tee *et al*. 1992; Smith *et al*. 1996). In contrast; M2PK is not expressed in adult parenchymal cells, only in hepatomas (Tian *et al*. 1997). Therefore M2PK is useful for identifying and counting oval cells when considered together with the unusual morphology and location of such cells.

As cytomegaloviruses are species specific, we have used murine (M) CMV infection of inbred mice to model HCMV disease in patients (Price & Olver 1996). Our earlier studies have shown that BALB/c mice infected with MCMV retain inflammatory cells in their livers for several months, whilst inflammation clears from C57BL/6 mice more rapidly (Olver *et al*. 1994). This study was undertaken to ascertain whether MCMV infection and the accompanying hepatitis leads to the induction of oval cell proliferation, and whether this response is affected by genetically determined resistance to persistent hepatitis.

## Materials and methods

### *Experimental animals and MCMV infection*

Specific pathogen-free female BALB/c and C57BL/6 mice were obtained from the Animal Resource Centre (Murdoch, Western Australia) at 8 weeks of age and maintained under rigorous barrier conditions. Regular serological monitoring confirmed that the holding room remained free of Sendai virus, *Mycoplasma pulmonis* and mouse hepatitis virus. Stocks of MCMV (K181(Perth) strain) were maintained by salivary gland passage in weaning female BALB/c mice and standardized as plaque-forming units (pfu) in mouse embryo fibroblasts (Allan & Shellam 1984). Mice were infected intraperitoneally with 0.5 times the amount of virus required to induce 50% mortality (0.5LD50). Hence BALB/c mice received $4\times10^4$ pfu, whilst C57BL/6 mice received $12\times10^4$ pfu in accordance with their greater resistance to acute infection. Groups of six infected and four control mice were assayed at each time point.

### *Tissue preparation for histology and immunocytochemistry*

The livers were perfused with PBS, removed, cut into 0.5 $cm^3$ fragments and immersed in Carnoy's fixative for 6 h. These were stored in 50% ethanol then dehydrated with graded ethanol (50, 70, 90, 100%) and cleared with Histoclear (Ajax chemicals, Melbourne, Australia) before embedding in paraffin wax. Tissue sections (6 $\mu$m) were cut and attached to slides previously coated with 2% 3-amino propyl triethoxysilane in acetone. Sections were dried at 37°C overnight, dewaxed with Histoclear, and rehydrated with graded ethanol (100, 90, 70%) and then water.

### *Histology*

Slides were stained with Harris' Haematoxylin followed by Eosin (H & E) using standard techniques.

*Immunocytochemistry*

To localize albumin, sections were treated with 3% hydrogen peroxide for 5 min to block nonspecific peroxidases; followed by TRIS-buffered saline (TBS) containing 20% normal swine serum. Sections were incubated overnight at 4°C in a humid chamber with the primary antibody antimouse albumin diluted in TBS. Antigen was detected with biotinylated F(ab′)2 fragment of swine antirabbit immunoglobulins (DAKO, Glostrup, Denmark), diluted in TBS (30 min), followed by peroxidase-conjugated streptavidin diluted in TBS (30 min) and 0.5 mg/ml diaminobenzidine (DAB) (Sigma, St Louis, MO, USA) substrate in 0.05 M TRIS HCl (10 min). The sections were rinsed with water, counterstained with haematoxylin and mounted in Depex (BDH, Poole, UK).

To identify inflammatory cells, an antibody cocktail containing rat monoclonal antibodies reactive with mouse CD8, Thy 1.2, CD4 and CD3 (T cell antigens) and B220 and J11d (B cell antigens) was used as a primary antibody, with normal rat serum as a negative control (Olver *et al*. 1994). Following treatment with the primary antibody, the sections were washed with 0.2% saponin in TBS (3x 15 min) and incubated with the secondary antibody, antirat IgG conjugated to horse radish peroxidase (HRP) (Amersham, Amersham, UK) diluted in 0.2% saponin in TBS. Sections were then washed, incubated with DAB substrate (15 min), rinsed in TBS and mounted in glycerol/gelatin (10 g gelatin, 60 ml DDW, 70 ml glycerin and 0.25 g phenol).

Goat antirabbit M2PK antibody (Rockland, Gilbertsville, PA, USA) was used to identify oval cells and antiproliferating cell nuclear antigen (PCNA)-biotin (Lenco Technologies, Ballwin, MO, USA) was used identify proliferating cells. Sections were treated with 2.5% periodic acid in TBS (5 min), TBS, 0.02% sodium borohydride in TBS (2 min), rinsed briefly in 0.2% saponin in TBS, then incubated with 10% foetal calf serum (FCS)/0.2% saponin in TBS (60 min). This was followed by anti-M2PK or anti-PCNA-biotin, diluted in 0.2% saponin in TBS (60 min). The sections were then washed with 0.2% saponin in TBS (3 × 15 min) and incubated with a donkey antigoat IgG – HRP conjugate (Rockland: USA) for M2PK or streptavidin-HRP (DAKO) for PCNA, diluted with 0.2% saponin in TBS (60 min). The sections were then washed in 0.2% saponin in TBS (3x 15 min), reacted with DAB (15 min), rinsed in TBS and mounted with glycerol/gelatin.

*In situ hybridization*

M2PK transcripts were localized in oval cells by *in situ* hybridization. The RNA probes were labelled with digoxygenin using the DIG RNA labelling kit (SP6/T7; Boehringer Mannheim, Mannheim, Germany). Sections were dewaxed in xylene (5 min), hydrated in ethanol (100%, 90%, 70%) 5 min each, rehydrated in DEPC treated double distilled water (DDW) (30 min) and treated with 0.2 M HCl (20 min). The slides were rinsed with PBS, treated with 0.3% Triton X-100 in PBS (15 min), rinsed with 0.1 M TRIS HCl pH 8 containing 50 mM EDTA and digested at 37°C with 15 mg/ml Proteinase K (Sigma) diluted in the same buffer (30 min). The sections were rinsed with 0.2% glycine, post fixed with freshly prepared 4% paraformaldehyde (5 min), and treated with 0.25% acetic anhydride containing 0.1 M triethanolamine (10 min). The sections were then prehybridized in a solution of 50% formamide/2 × SSC, for 2 h at 37°C in a damp chamber.

Hybridization was performed using the DIG labelled M2PK SP6 (antisense probe), M2PK T7 (sense probe). These were adjusted to 0.04 ng/ml and denatured by boiling. The probes were diluted in 0.01 M Tris HCl pH 7.5, Denhardt's solution (polyvinylpyrrolidine 2% w/v, bovine serum albumin 2% w/v & Ficoll 400 2% w/v in DDW), 2x SSC, 50% formamide, 0.5% SDS, 250 mg/ml single stranded DNA in DEPC treated DDW. Sections were incubated with the probes at 37°C overnight in a humid chamber. This was followed by washes of increasing stringency; twice in 2x SSC at room temperature (15 min), twice in 0.1 × SSC at 42°C (20 min), followed by a 10 minute wash with 0.1 M TRIS HCl pH 7.5 containing 0.15 M NaCl.

The sections were then blocked for 30 min with 5% blocking reagent (Boehringer Mannheim) in 0.1 M TRIS HCl pH 7.5 containing 0.15 M NaCl, then quickly rinsed in the same buffer. To detect the bound probe, the sections were incubated with anti digoxigenin antibody (Boehringer Mannheim) diluted 1 : 500 in the above blocking solution (2 h). Sections were washed then incubated with a buffer (0.1 M TRIS HCl pH 9.5/0.1 M NaCl/0.05 M $MgCl_2$) at 37°C in a humid chamber. The colour substrate solution, 4.5 μl/ml nitroblue tetrazolium salt (NBT) and 3.5 μl/ml X-phosphate (Boehringer Mannheim) in buffer was then applied and incubated at 37°C overnight in the dark in a humid chamber. The sections were rinsed with 10 mM TRIS HCl and 1 mM EDTA (pH 8), and mounted in glycerol/gelatin.

*Staining for viral antigens*

Sections were dewaxed, rehydrated, blocked with 3% hydrogen peroxide followed by 20% normal goat serum (NGS) in TBS, and treated with anti-MCMV hyperimmune serum (generated in BALB/c mice) diluted in

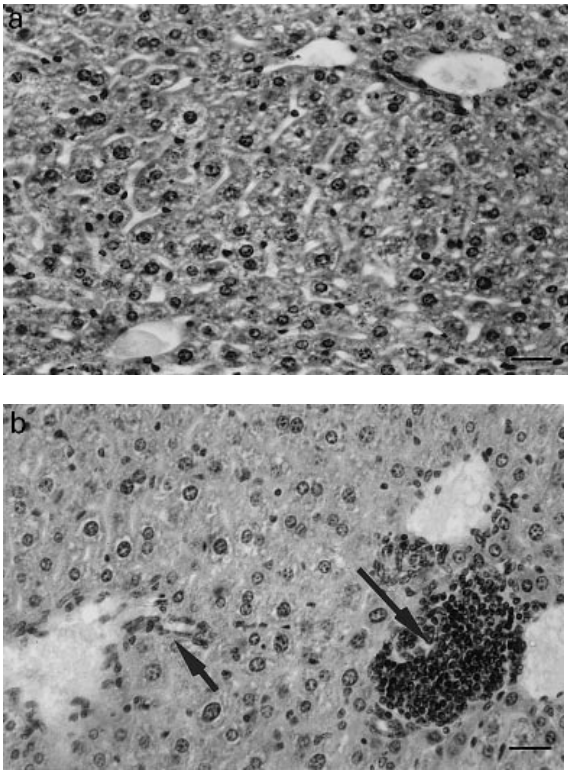


**Figure 1.** Histology of MCMV-infected liver. H & E stained liver sections from (a) control mouse and (b) MCMV-infected mouse12 months after infection. A large cluster of inflammatory cells around a central vein (b – large arrow) and putative oval cells adjacent to a portal area (b – small arrow) are indicated. Magnification bar represents 100 $\mu$m.

TBS/5% NGS (60 min). The slides were then washed in TBS twice, and rabbit antimouse peroxidase (DAKO, Denmark) diluted in TBS/5% NGS was added. Slides were then washed, treated with DAB substrate, washed, counterstained with haematoxylin and mounted in Depex. Positive cells in sections from mice infected for 3–7 days stained parallel with this study displayed a brown precipitate with or without typical CMV inclusions.

*Quantification of inflammatory cells and oval cells*

Inflammatory cells and oval cells were stained with the inflammatory cell antibody cocktail or M2PK antibody, respectively, and 5 fields were scored for each liver. The number of positive cells was obtained by counting the number of cells that stained positively and expressed as a percentage of the number of hepatocytes in each field. Inflammatory cells were counted over the entire liver lobule as they were evenly distributed, while oval cells were scored over the portal triad. As scores for individual animals within groups did not vary markedly, the counts for each group were pooled and expressed as mean $\pm$ SE.

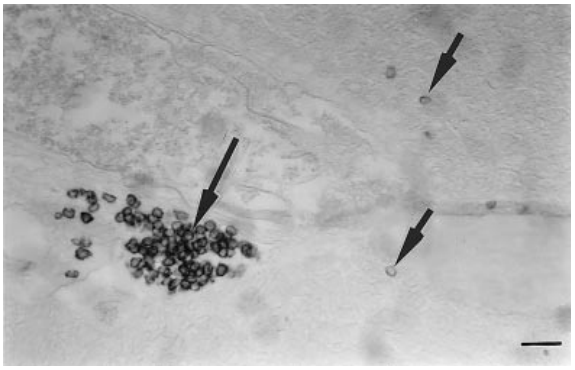

**Figure 2.** Arrangement of inflammatory cells in MCMV-infected liver. Inflammatory cells are visualized by staining with inflammatory cell antibody cocktail. They are either arranged in large clusters located adjcent to the central vein (large arrow) or dispersed as individual cells throughout the liver parenchyma (small arrows). Magnification bar represents 100 $\mu$m.

## Results

*General features of the liver following MCMV infection*

To assess the histopathological changes in MCMV infected livers, H & E stained sections collected 4–12 months after infection were examined. No cells expressing viral antigens remained at any time points assayed, as expected from previous studies (Olver *et al.* 1994). There were no obvious changes in the hepatocytes 4–12 months after MCMV infection in BALB/c (Figure 1a,b) or C57BL/6 (data not shown) mice. In contrast, there was a substantial increase in the number of small cells at all stages studied. These cells were about an eighth of the diameter of hepatocytes and were of two types. Cells resembling oval cells in shape, with ovoid nuclei and scant cytoplasm were mainly located in the periportal regions. The other small cells were round with basophilic nuclei and resembled inflammatory cells. These were most abundant around the blood vessels (Figure 1b).

To assess lymphocytic infiltration, sections were stained with antibodies recognizing surface markers on B and T cells. Livers from uninfected mice of both strains contained inflammatory cells that were scattered or in small clusters (Figure 2). These displayed moderate increases from 4 to 12 months of age. However, numbers

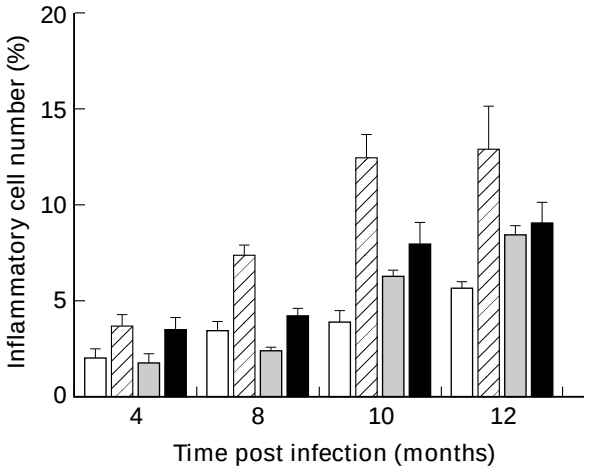


**Figure 3.** Inflammatory cell numbers in MCMV-infected liver. Comparison between control and MCMV-infected mice of the BALB/c and C57BL/6 strains as a function of time after infection. □ Control BALB/c; ▨ CMV BALB/c; ■ Control C57BL/6; ■ CMV C57BL/6 The number of inflammatory cells is expressed as a percentage of hepatocytes. Error bars represent ± SE.

of infiltrating lymphocytes in livers from BALB/c mice trebled by 10 months post infection (Figure 3). This marked increase was not seen in C57BL/6 mice.

*Oval cells*

Oval cells were initially identified in tissues from BALB/c mice by *in situ* hybridization using an antisense M2PK SP6 DIG labelled RNA probe. M2PK transcripts were localized as dark purple precipitates in the cytoplasm of cells with the morphology of oval cells (Figure 4). A sense probe (M2PK T7) used as a control yielded no positive staining. Unfortunately with this method some sinosiodal staining is possible due to the reactivity of the probe, however, this was at a low level in comparison to the positive cells. Most oval cells were found in periportal regions mainly around the vessels, but some were observed in the central regions and in two sections these formed tracks of cells radiating from a portal area. Numbers of oval cells in the portal regions increased progressively in BALB/c mice infected with MCMV (Figure 5). Tissues from uninfected mice contained few oval cells.

To confirm these results, oval cell numbers were determined independently by immunocytochemistry using an M2PK antibody. The morphology and pattern of staining of these cells is shown in Figure 6. Oval cells characterized by ovoid nuclei and staining positively for

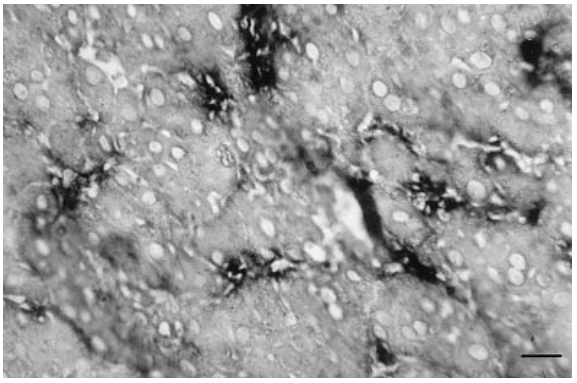

**Figure 4.** Localization of oval cells by *in situ* hybridization using a M2PK-DIG labelled probe. Oval cells located periportally in liver from BALB/c mice infected for 12 months with MCMV sections show strong hybridization to the probe (dark stain). Magnification bar represents 100 $\mu$m.

M2PK were usually located periportally and in some instances formed duct like structures (Figure 6a). Bile duct cells were negative as were hepatocytes (Figure 6b). To assess genetic differences, tissue sections from BALB/c and C57BL/6 mice were studied. Following MCMV infection, more oval cells were evident in BALB/c than C57BL/6 tissues (Figure 7). Numbers also increased in both strains with age, but these changes were minor relative to the effect of MCMV in BALB/c mice.

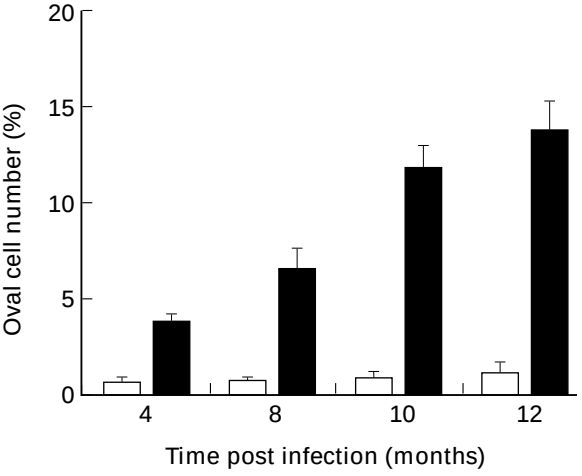


**Figure 5.** Induction of oval cells following MCMV-infection in BALB/c mice. □ Control BALB/c; ■ CMV BALB/c. Oval cells were identified by *in situ* hybridization with a M2PK RNA antisense probe. The number of oval cells is expressed as a percentage of hepatocytes. Error bars represent ± SE.

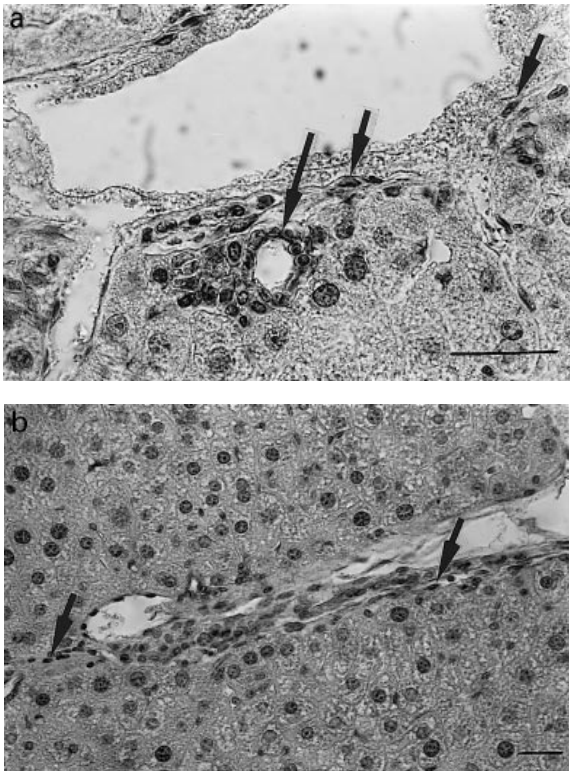


**Figure 6.** Localization of oval cells by immunocytochemical staining using an anti-M2PK antibody. Oval cells located periportally stain positively for M2PK in liver sections from BALB/c mice 12 months post infection (small arrows – a and b) adjacent to a portal vein. Some have formed a pseudoduct (a – large arrow). Magnification bar represents 100 $\mu$m.

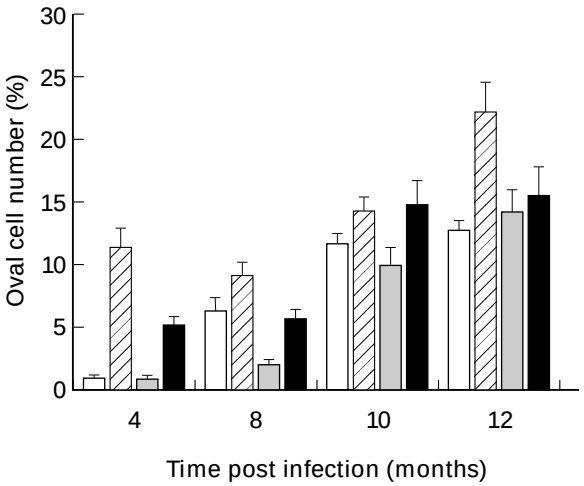


**Figure 7.** Induction of oval cells following MCMV-infection in BALB/c and C57BL/6 mice. □ Control BALB/c; ▨ CMV BALB/c; ■ Control C57BL/6; ■ CMV C57BL/6. Oval cells were identified morphologically and by immunocytochemical staining with anti-M2PK. The number of oval cells were scored in periportal regions and expressed as a percentage of hepatocytes. Error bars represent ± SE.

In addition to M2PK positive oval cells, a few M2PK positive small hepatocyte-like cells were identified by *in situ* hybridization, in two MCMV infected BALB/c livers (data not shown). These cells had a morphology similar to a hepatocyte but were only half the diameter and stained less intensely than oval cells. These are likely to be maturing oval cells. To examine this further, tissues of BALB/c mice were stained with an antibody recognizing mouse albumin. All hepatocytes and oval cells, as well as the small hepatocytes stained positively, while inflammatory cells were negative. The presence of albumin in oval cells suggests they are differentiating along the hepatic lineage (Becker *et al.* 1996).

### *Proliferating cells*

As oval cells were most numerous in BALB/c mice, this strain was used to assess the role of cell proliferation in their accumulation. C57BL/6 mice were then examined at the 4 and 8 month time periods for proliferating cells. At all time points hepatocytes and oval cells staining positively for PCNA were present in both the central and periportal regions of liver sections prepared from MCMV infected BALB/c mice (Figure 8). In control tissues proliferating hepatocytes were observed rarely and proliferating oval cells were not seen. Similar results were obtained with C57BL/6 mice as PCNA staining was seen at similar levels.

## Discussion

HCMV has been associated with cervical and nasopharyngeal carcinomas, but has yet to be ascribed a primary role in hepatic cancers (Huang & Kowalik 1993). The interpretation of data is complicated by the high seropositivity rate for HCMV in the general population and the plethora of viral, dietary and chemical cofactors that must be evaluated. However, several studies suggest that HCMV, and hence by inference MCMV infection, can lead to cancer (Ho 1991; Ikeda *et al.* 1993; Rosenthal & Choudhury 1993). In animal models of hepatocarcinogenesis (Sell & Dunsford 1989; Thorgeirsson 1995), cellular changes may occur in the liver before the development of carcinomas. These include the formation of oval cells which proliferate extensively, initially in the

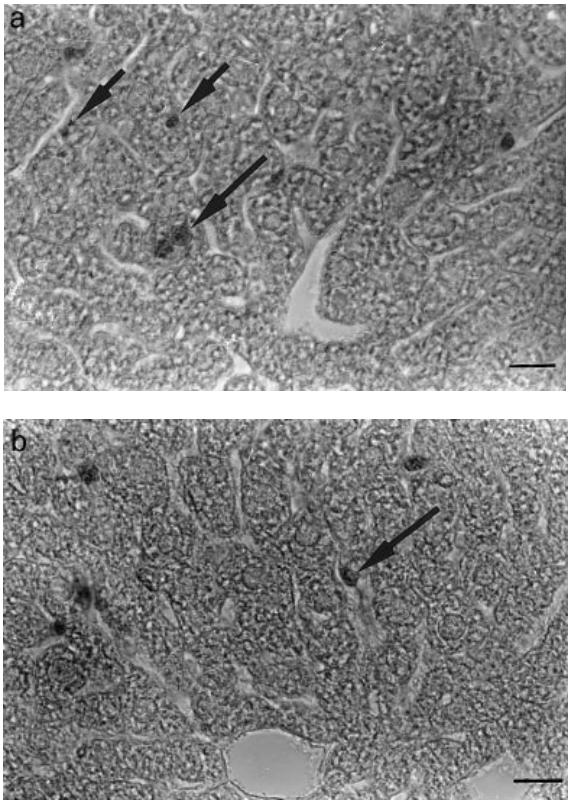


**Figure 8.** Proliferating cell nuclear antigen (PCNA) positive cells in BALB/c 8 month post MCMV-infected liver. PCNA positive hepatocytes (large arrow) are observed throughout the liver lobule around the portal tract (a) as well as the central vein (b). Oval cells (small arrows) are mainly observed around the portal tracts (a). Magnification bar represents 100 $\mu$m.

periportal areas and later infiltrate the liver parenchyma leading to significant liver remodelling. There is compelling evidence to support a link between the oval cells and hepatoma in these models (Sell & Dunsford 1989; Thorgeirsson 1995; Smith *et al*. 1996; Tee *et al*. 1996). The effect of cytomegaloviruses on oval cell accumulation has not previously been assessed and is addressed here using inbred mice infected with MCMV. Infection is known to induce hepatic inflammation which persists after the clearance of replicating virus and viral antigen. Inflammatory cells include CD4+ and CD8+ cells, and are more persistent in BALB/c than C57BL/6 mice (Olver *et al*. 1994).

Histological examination of the livers confirmed that inflammatory lymphocytes accumulate in both C57BL/6 and BALB/c mice after MCMV infection, but numbers were significantly higher in BALB/c mice. There were also small ovoid-shaped cells, particularly around the periportal regions at all stages examined in MCMV infected mice. Increases in numbers of inflammatory and putative oval cells in control livers were less dramatic and are attributed to advancing age.

Oval cells express oncodevelopmental proteins that are no longer expressed in mature hepatocytes (Tee *et al*. 1994). Of these, the foetal isoenzyme of pyruvate kinase (M2PK) is not expressed by inflammatory cells. Hence oval cells can be distinguished from adult hepatocytes and inflammatory cells by their expression of M2PK and morphology. Positive staining with M2PK supports the view that the oval-shaped cells are indeed oval cells and resemble foetal liver cells (Tian *et al*. 1997). The oval cells also stained positively for albumin, which is expressed in foetal and adult hepatocytes, but not inflammatory cells.

The presence of M2PK positive small hepatocytes in the livers of MCMV infected mice suggests that some of the oval cells mature and contribute to liver regeneration. Such cells have been described in normally developing liver (Tian *et al*. 1997) and in CDE-treated liver (Tee *et al*. 1994).

The presence of progenitor cells which are oval cell-like and stain positively for M2PK and PCNA in serial sections has been demonstrated in newborn liver (unpublished observation). It is likely that these cells contribute to the rapid growth of the liver during this period of development. Oval cells have also been induced to proliferate in adult rat livers treated with carcinogens, where they are thought to participate in liver regeneration (Brill *et al*. 1993). Here the proliferation of oval cells in MCMV infected livers (demonstrated by PCNA staining) provides a mechanism for the observed increase in oval cell numbers. All livers collected after MCMV infection contained PCNA positive hepatocytes and oval cells suggesting that oval cells and hepatocytes contribute to liver regeneration in MCMV infected liver. Control tissues had fewer proliferating cells, and these were all hepatocytes.

The correlation between numbers of oval cells and inflammatory cells in the two strains of mice investigated in this study suggests a common regulatory mechanism. Critical events may occur less than 4 months after infection as the difference in oval cell numbers between control and infected mice was apparent at this time. Hence it is possible that the difference between mice of the two strains may arize during acute infection. The virus is cleared more rapidly from C57BL/6 mice in the first week after infection as they exhibit a more effective NK response in the liver (Olver & Price 1998). The induction of oval cells may

be the result of the virus causing severe liver damage and/or the accompanying infiltration of inflammatory lymphocytes.

Alternatively the association between MCMV infection and oval cell expansion may be mediated by viral replication in oval cells or their precursors in the liver or bone marrow. Viral replication occurs at both sites in cells of several lineages including monocytes, fibroblasts, endothelial cells and hepatocytes (Price & Olver 1996). Replication in oval cells has never been addressed but could influence the metabolism of p53, enhancing the proliferation of oval cells and increasing the possibility of their transformation into malignancy.

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