Cytomegalovirus-induced pneumonitis and myocarditis in newborn mice

A model for perinatal human cytomegalovirus infection

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Summary. Genetically determined resistance to the lethal effects of infection with murine cytomegalovirus (MCMV) has been reported previously in adult and newborn mice. We examined the pathogenesis of MCMV infection in resistant (CBA, H-2^k) and susceptible (BALB/c, H-2^d) mice infected intraperitoneally on the day of birth. BALB/c mice developed a severe interstitial pneumonitis and myocarditis 10 days post-infection. Their pulmonary and cardiac tissues contained high MCMV titres and large numbers of MCMV-antigen positive cells. MCMV also infected the endothelial and myointimal cells of the coronary arteries in newborn BALB/c mice. Only limited infection and pathological changes were seen in CBA mice. Since the severe disease in BALB/c mice resembles the pneumonitis and less frequently reported myocarditis observed after perinatal HCMV infection, the newborn mouse model will be useful for studying the consequences and treatment of such infections, the influence of the host genotype on disease severity and the possible association between perinatal HCMV infection and atherosclerosis.

Introduction

While the majority of human cytomegalovirus (HCMV) infections are not clinically apparent, symptomatic HCMV infections can occur in immunocompromised individuals, infants infected in-utero and low birth weight infants infected perinatally with HCMV. Full-term healthy infants perinatally infected with HCMV are generally asymptomatic [18, 21], although transient symptoms such as pneumonitis, hepatosplenomegaly and lymphadenopathy have been reported [11]. Infection can occur at birth from HCMV in cervical excretions or postnatally from breast milk [16]. However, premature and low birth weight

neonates are at greater risk of developing serious and fatal disease after infection with HCMV. Transfusion-acquired HCMV infections are a significant risk to seronegative low birth weight infants who receive blood from seropositive donors [1, 3, 27]. Serious disease, in which pneumonitis is one of the most severe manifestations, occurs in 50% of these infants and a mortality rate of 40% has been reported [27]. In addition, low birth weight and preterm infants acquiring HCMV naturally from their mothers may also develop a symptomatic infection [8, 28].

It is not understood why some seronegative compromised infants should develop severe disease manifestations while others have an asymptomatic infection after exposure to HCMV. Similarly it is not known why a proportion of healthy full-term infants develop pneumonitis after HCMV infection, while the majority remain asymptomatic. In the adult mouse model for CMV infection, restistance to murine CMV (MCMV) lethality is genetically controlled by genes associated with the H-2 complex [7]. Resistance is conferred by the H-2^k haplotype, while mouse strains having other haplotypes (e.g. H-2^b, H-2^d) are relatively susceptible. Genetically-determined resistance to lethal MCMV infection has also been observed in newborn mice [26]. We have used newborn mice of a resistant (H-2^k) and susceptible (H-2^d) strain to examine the effect of the host genotype on MCMV-replication and histological damage in a variety of organs, including the lung, after infection with MCMV on the day of birth. The relevance of this model for the study of pneumonitis in infants infected perinatally with HCMV is described. We also report that myocarditis is a major feature of perinatal MCMV infection in BALB/c mice and discuss the implications of this finding, including its association with viral invasion of coronary endothelial and myointimal cells.

Materials and methods

Virus

A stock of the Smith strain of MCMV was used for all experiments. The origin and maintenance of MCMV by salivary gland passage has been described elsewhere [2]. To reduce the requirement for serial dilutions, a working stock of 1.5×10^3 plaque-forming units (PFU)/0.05 ml was prepared and stored in a number of aliquots in liquid nitrogen.

Animals

Specific pathogen free, advanced pregnant mice were obtained from the Animal Recources Centre, Murdoch, Western Australia and subsequently housed under minimal disease conditions. Mice used for histopathological studies were housed under isolated conditions in boxes with filter hoods as an extra precaution to keep them free from respiratory pathogens. Inbred BALB/cLac (H-2^d) and CBA/CaH(H-2^k) mice were used. Random serological screening established that the holding colony remained free of mouse hepatitis virus, Sendai virus and *Mycoplasma pulmonis*. In addition, samples of lung tissue were taken from MCMV-infected and control experimental animals, and the tissue extract found to be negative for bacterial growth on blood and chocolate agar.

Inoculation of newborn mice

Newborn mice were inoculated intraperitoneally (i.p.) on the day of birth with the appropriate number of PFU of MCMV diluted in phosphate buffered saline (pH 7.2, 333 mmol/kgH$_2$O) plus 0.5% foetal calf serum as described previously [26]. An inoculum volume of 0.05 ml/mouse was used. Control mice were inoculated in a similar manner with diluent alone. Only litters with 4 to 7 mice were used.

Determination of MCMV lethality in newborn mice

The i.p. dose of MCMV which killed 50% of the animals (LD$_{50}$) was calculated in newborn BALB/c and CBA mice as described previously [26]. Three litters of each strain were inoculated per dilution and observed daily for deaths. A series of two fold dilutions of MCMV covering a range of 0 to 100% mortality was used.

Measurement of MCMV titres in various organs

Three litters of CBA and BALB/c mice were harvested on each of days 4,7 and 10 post-infection (p.i.) with 6PFU of MCMV. This dose was lethal for 95% of BALB/c mice, which died at $\geqslant$ 10 days p.i., and rarely lethal for CBA mice ($<$ 5%). Spleens, livers, hearts and lungs were removed aseptically, pooled from each litter and weighed. Thus, each litter of mice gave rise to one sample of each organ. Organs were homogenized and the levels of infectious MCMV were determined by a plaque assay in mouse embryo fibroblasts as described previously [2], modified to use 24-well trays (Costar, U.S.A.) seeded with 2×10^5 cells/well. The results are expressed as the PFU of MCMV per g of tissue.

Histological examination and viral antigen expression

Following the experimental protocol described above, organs were fixed in Bouins fluid for 15 h and transferred to Tris-buffered saline (10mM Tris, 500 mM NaCl, pH 7.6) prior to processing and embedding in paraffin wax. In addition, control tissues were harvested at the stated periods from BALB/c and CBA mice inoculated with diluent alone. Sections were stained with haematoxylin and eosin (H & E) for histopathological studies. Immunoperoxidase histochemistry employing BALB/c hyperimmune antiserum to MCMV was used to detect MCMV-antigen positive cells as described previously [4]. Briefly, dewaxed sections were blocked with 0.3% hydrogen peroxide and treated with 20% normal goat serum. They were then incubated with the hyperimmune antiserum to MCMV followed by a peroxidase-conjugated rabbit antibody to mouse immunoglobulin (Dakopatts, Glostrup, Denmark). Diaminobenzidine (Sigma, St. Louis, MO, U.S.A.) substrate was added resulting in a dark brown precipitate in MCMV-antigen positive cells. The slides were counterstained with haematoxylin. When normal mouse serum was used in place of the hyperimmune antiserum in the above procedure, a positive reaction was not observed in sections known to be MCMV-antigen positive.

Statistics

Errors are expressed as the standard error of the mean (SEM). The Student's t-test was used for statistical analyses. A p value $\leqslant$ 0.05 was taken as being significant.

Results

Genetically controlled resistance to lethal MCMV infection in newborn mice

Newborn CBA mice were 28-fold more resistant than newborn BALB/c mice when assessed by LD$_{50}$ titres (Table 1). However, in both cases, the newborn

mice were 2.5×10^4-fold more susceptible than their adult counterparts (Table 1).

Replication of MCMV in newborn mice

In all organs examined, BALB/c mice had significantly higher MCMV titres than CBA mice at day 7 and 10 p.i. (Fig. 1). The lungs were found to have the highest virus titres in both CBA and BALB/c mice compared to other organs at day 7 and 10 p.i. The hearts and spleens also had relatively high levels of

Table 1. Genetically determined resistance to MCMV lethality in newborn mice

Mouse strain	H-2 haplotype	Approximate i.p. dose for 1 LD$_{50}$ (PFU of MCMV)	
		Newborns[a]	Adults[b]
BALB/c	d	2	5×10^4
CBA	k	55	1.4×10^6

[a] Mice were inoculated i.p. on the day of birth with various concentrations of MCMV

[b] Adapted from Shellam and Flexman (1986) and A. Scalzo (pers. comm.)
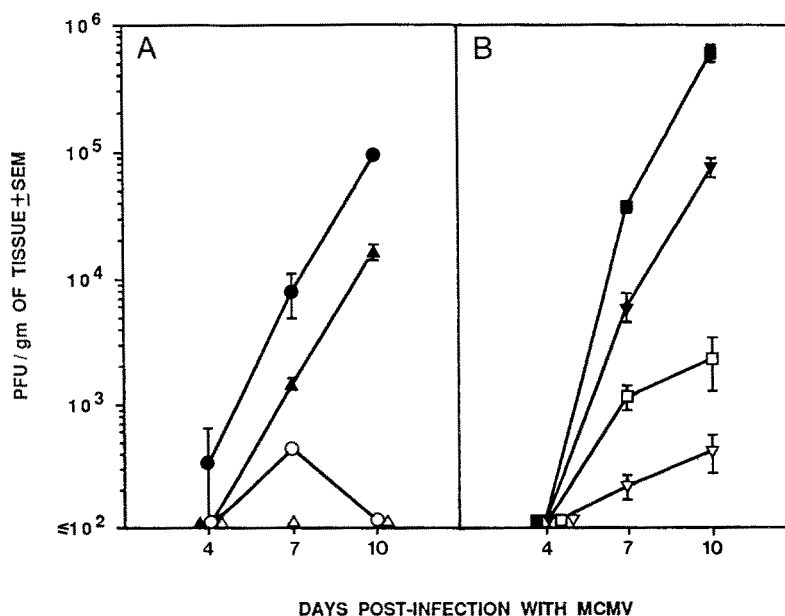

Fig. 1. Kinetics of MCMV replication in various organs after i.p. inoculation of newborn mice with 6 PFU of MCMV. **A** BALB/c spleen (●) and liver (▲); CBA spleen (○) and liver (△). **B** BALB/c heart (▼) and lung (■); CBA heart (▽) and lung (□). Results are expressed as the mean of three samples ± SEM

MCMV in the susceptible BALB/c strain, while the liver had the least amount of virus (Fig. 1). BALB/c mice died from day 10 p.i.

Effect of MCMV infection on body weight and organ weight

Individual control and infected mice were weighed when harvested 10 days after inoculation. A minimum of 18 individual weights was used for each group. The mean weight of infected BALB/c mice was significantly lower than that of control BALB/c mice (p ≤ 0.0005, Table 2). Infected CBA mice were slightly runted compared to control CBA mice (0.005 < p < 0.01).

The organs harvested from three control and three infected BALB/c and CBA litters 10 days after inoculation were weighed as a pool for each litter and the mean individual organ weight was calculated. The organ weights in infected mice were either not significantly altered or were reduced compared to controls (for BALB/c liver, p ≤ 0.0005; BALB/c lungs, 0.005 < p < 0.01; CBA lungs, 0.0005 < p < 0.005; Table 2). However, when the organ weights were expressed as a percentage of the body weight and compared in infected and control mice, there was an increase in infected BALB/c mice for the spleen and heart and to

Table 2. Effect of MCMV infection on body and organ weights[a]

	BALB/c		CBA	
	control	infected	control	infected
Mean body weight (g) ± SEM[b]	6.35 ± 0.26	3.58 ± 0.21	5.62 ± 0.05	5.24 ± 0.19
Mean organ weight (mg) ± SEM				
Spleen	47 ± 2	46 ± 3	36 ± 0	37 ± 6
Liver	242 ± 4	165 ± 5	243 ± 5	254 ± 40
Heart	56 ± 1	57 ± 3	63 ± 4	58 ± 14
Lung	120 ± 10	78 ± 1	115 ± 3	90 ± 10
Organ weight as a % of mean body weight[c]				
Spleen	0.74%	1.28%	0.64%	0.71%
Liver	3.81%	4.61%	4.32%	4.85%
Hart	0.88%	1.59%	1.12%	1.11%
Lung	1.89%	2.18%	2.05%	1.72%

[a] Mice were inoculated i.p. with 6 PFU of MCMV or diluent alone on the day of birth and sacrificed 10 days p.i.

[b] A minimum of 18 individual weights were used for each group

[c] To calculate the organ weight as a % of body weight, the mean organ weights and body weights were used

a lesser extent for the liver and lungs, with only minor changes in CBA mice (Table 2).

Histology and MCMV antigen expression

Organs were removed from mice for histological examinations 4, 7 or 10 days after infection with 6 PFU of MCMV on the day of birth, or from age-matched uninfected controls.

No tissue damage, inflammation or MCMV-antigen-positive cells were seen in tissues from uninfected mice.

Heart

Infected BALB/c mice developed a severe endocarditis and myocarditis and both were more severe in the atria than in the ventricles. Lesions first appeared seven days p.i. They consisted predominantly of enlarged endocardial and subendocardial cells and cardiac myocytes, many of which (especially the sub-endocardial cells) displayed amphophilic intranuclear inclusions characteristic of CMV infection and were surrounded by macrophages, polymorphonuclear leucocytes and lymphocytes (Fig. 2a). By 10 days p.i., these lesions were more numerous and larger than those seen at earlier times (Fig. 2b). Necrotic cells and cells bearing intranuclear inclusions were common in these lesions. In addition, similar foci were found in the myocardium while scattered endothelial and myointimal cells of the coronary arteries were enlarged and possessed amphophilic intranuclear inclusions (Fig. 2c, d). The hearts of infected CBA mice were much less affected than in the BALB/c mice, with only a few scattered aggregates of macrophages, granulocytes and lymphocytes in the subendocardial region and myocardium by day 10 p.i.

The number of lesions per microscopic field in BALB/c and CBA hearts 10 days p.i. were counted and the results are shown in Table 3. As well as being much smaller, the number of inflammatory lesions per microscopic field of view in CBA hearts was significantly lower than in BALB/c mice 10 days p.i. ($p \leqslant 0.0005$, Table 3).

MCMV-antigen positive cells were not detected in the hearts of infected

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Fig. 2. Histopathology of the heart after MCMV infection of newborn mice. a Atrium of infected BALB/c mouse 7 days p.i. Note the subendocardial aggregation of cytomegalic cells surrounded by inflammatory cells (H & E, × 160). b Ventricle of infected BALB/c mouse 10 days p.i. Many inflammatory foci punctuate the myocardium (H & E, × 160). c Coronary artery of infected BALB/c mouse 10 days p.i. Two cytomegalic myointimal cells are present (arrow) (H & E, × 400). d Coronary artery of infected BALB/c mouse 10 days p.i. stained for MCMV antigen using the immunoperoxidase technique. Dark reaction product is present in endothelial and myointimal cells (× 400). e Atrium of infected BALB/c mouse 7 days p.i. stained for MCMV antigen using the immunoperoxidase technique. There is intense reaction product in two endocardial cells (× 400)
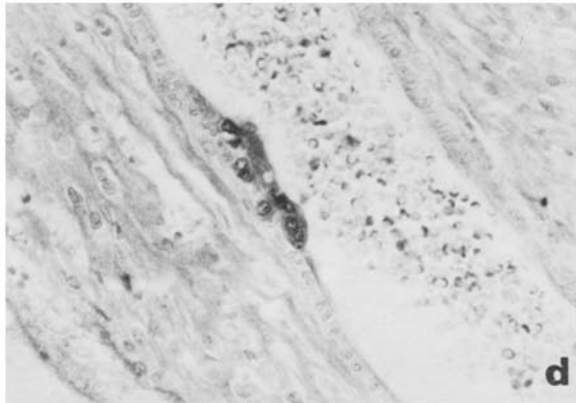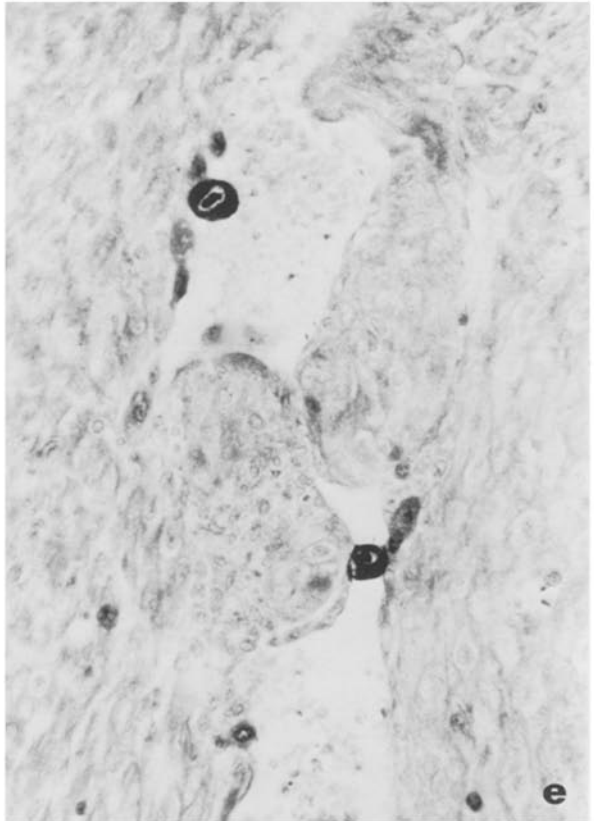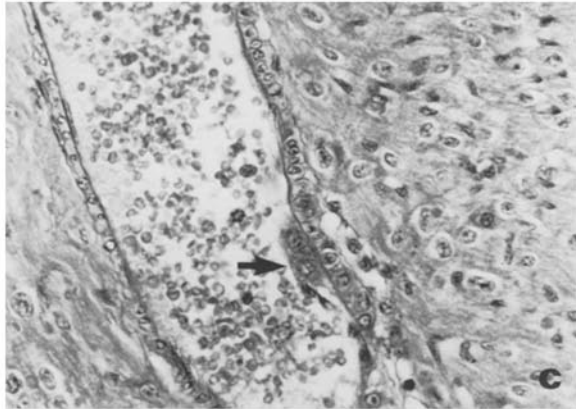
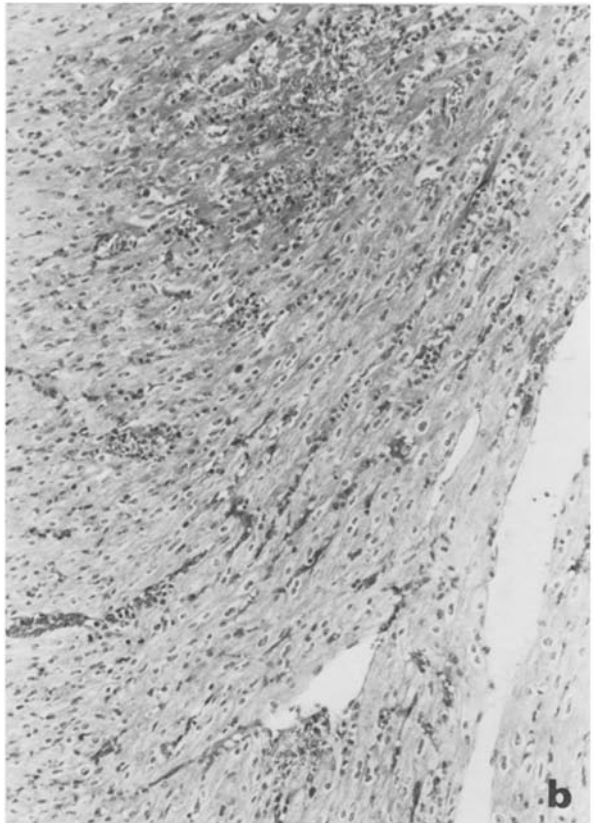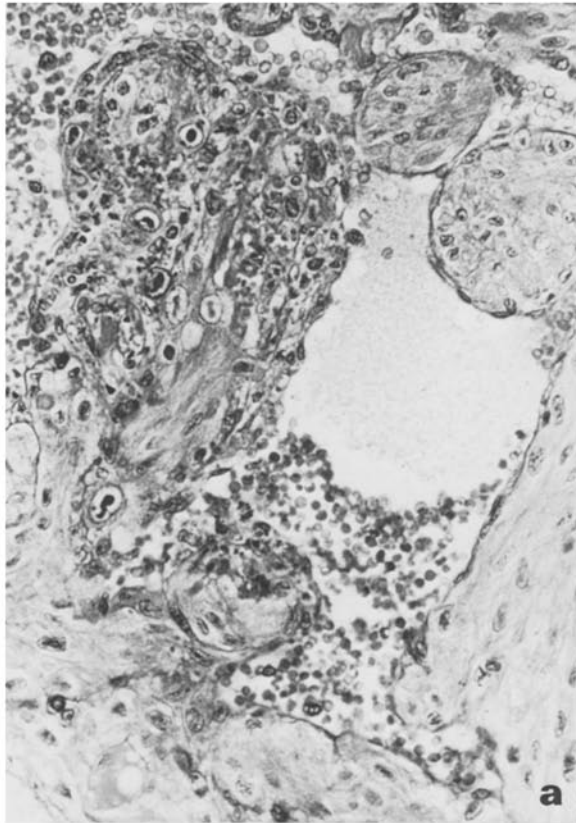**Table 3.** Comparison of BALB/c and CBA hearts and lungs 10 days p.i. with MCMV[a]

		BALB/c	CBA
Hearts	number of distinct inflammatory lesions per microscopic field ($\times 40$) $\pm$ SEM	7.56 ± 0.65	0.40 ± 0.13
	number of MCMV-antigen positive cells per microscopic field[b] ($\times 40$) $\pm$ SEM	20.19 ± 4.70	0.12 ± 0.08
Lungs	number of distinct inflammatory lesions per microscopic field ($\times 25$) $\pm$ SEM)	15.90 ± 1.06	1.75 ± 0.28
	number of MCMV-antigen positive cells per inflammatory focus $\pm$ SEM	17.27 ± 2.41	0.79 ± 0.32

[a] Mice were inoculated i.p. on the day of birth with 6 PFU of MCMV or diluent alone. Evaluation was performed 10 days p.i. as this was the time of maximum involvement of these organs. A minimum of 15 microscopic fields from the organs of 9 individual mice were scored for each parameter

[b] Antigen positive cells in the hearts were not always within inflammatory foci

BALB/c mice until 7 days p.i. and were numerous by day 10 p.i. at the sites indicated above. Positive cells were not always associated with inflammatory lesions. MCMV antigen was detected in endocardial (Fig. 2e) and subendocardial cells in the abnormal areas as well as myocytes, endothelial cells and myointimal cells of the coronary arteries in infected BALB/c mice (Fig. 2d). However, in infected CBA animals, MCMV-antigen-positive cells were not seen 4 and 7 days p.i. and were rarely found 10 days p.i. The number of antigen positive cells per microscopic field was significantly lower in the hearts of CBA mice than BALB/c mice 10 days p.i. (p $\leqslant$ 0.0005, Table 3).

Lungs

Infected BALB/c mice developed a severe interstitial pneumonitis. It began 7 days p.i. with scattered aggregates of granulocytes and macrophages which surrounded enlarged interstitial cells, several of which possessed amphophilic intranuclear inclusions (Fig. 3a). By 10 days p.i. these interstitial foci had en-

Fig. 3. Histopathology of the lung after MCMV infection of newborn mice. **a** Lung of infected BALB/c mouse 7 days p.i. A focal interstitial pneumonitis is evident (H & E, $\times$ 400). **b** Lung of infected BALB/c mouse 10 days p.i. Cytomegalic inclusion-bearing cells, necrotic cells and inflammatory cells contribute to the pulmonary lesions (H & E, $\times$ 400). **c** As in **b**. A thrombus is present in the lumen of an arteriole while the wall shows evidence of necrosis and leucocytic invasion (H & E, $\times$ 400). **d** Lung of infected BALB/c mouse 7 days p.i. stained for MCMV antigen using the immunoperoxidase technique. The reaction product is seen in an arteriolar endothelial cell ($\times$ 400). **e** Lung of infected BALB/c mouse 10 days p.i. stained for MCMV antigen using the immunoperoxidase technique. Reaction product is present in perivascular cells ($\times$ 400)
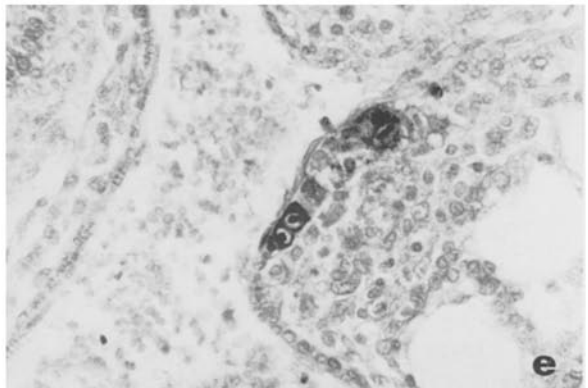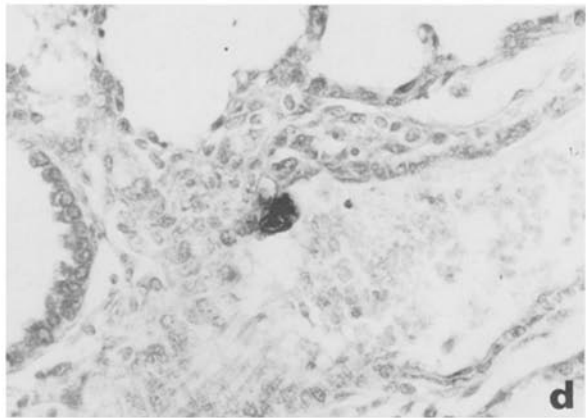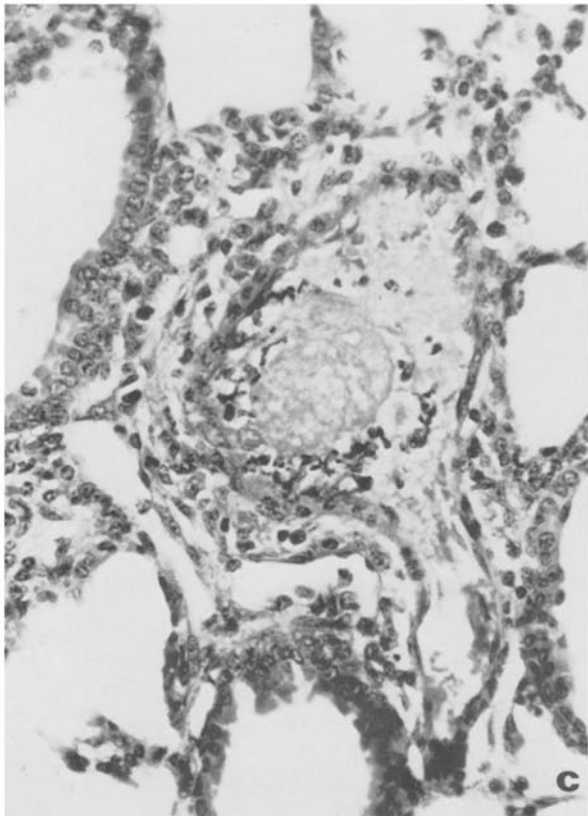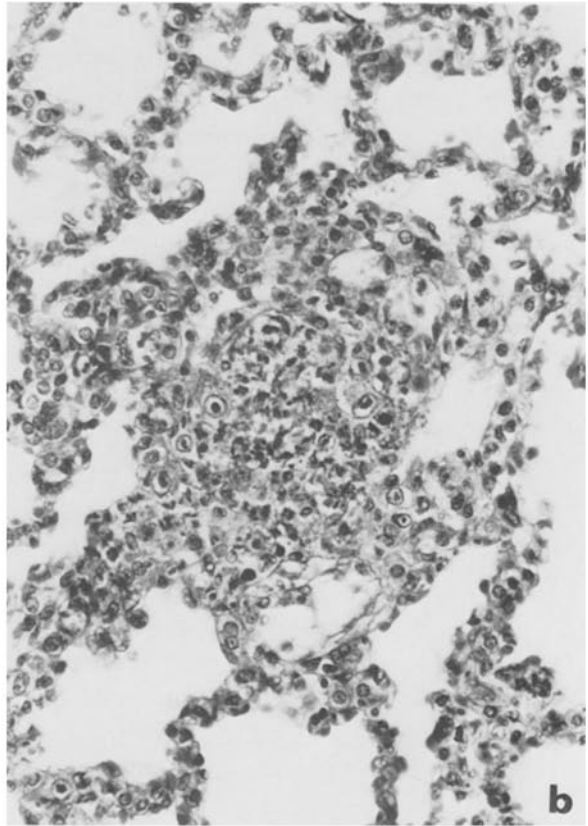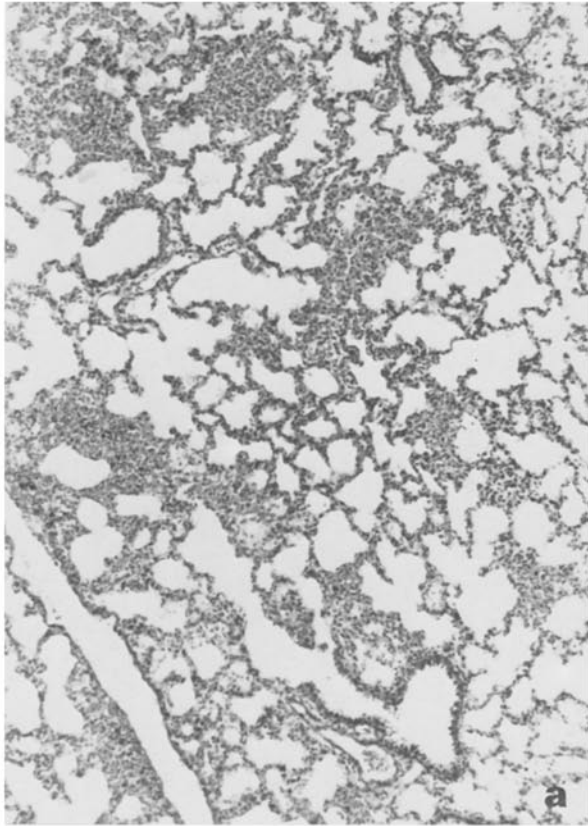
larged and contained many necrotic cells and nuclear-inclusion bearing cells (Fig. 3b). Approximately one third of the pulmonary parenchyma was involved. Often these foci surrounded small arteries and arterioles; the latter were sometimes thrombosed as the lesion progressed through the vascular wall (Fig. 3c). Occasionally a few arteriolar endothelial cells were enlarged and possessed intranuclear amphophilic inclusions. Unlike the BALB/c mice, CBA mice displayed only mild interstitial pneumonitis which began 7 days p.i. with a few scattered aggregates of granulocytes and macrophages in the pulmonary interstitium. By day 10 p.i., these inflammatory foci had enlarged a little and a few contained enlarged interstitial cells with amphophilic intranuclear inclusions. These inflammatory foci were much smaller than those observed in BALB/c lungs. The number of inflammatory foci per microscopic field in CBA lungs was significantly lower than in BALB/c lungs 10 days p.i. ($p \leqslant 0.0005$, Table 3).

MCMV-antigen positive cells were detected in the lungs of infected BALB/c mice by day 7 p.i. and were numerous within the inflammatory lesions at day 10. MCMV antigen was demonstrated in the nuclei of interstitial cells and occasionally in those of the arteriolar endothelium and adjacent perivascular cells (Fig. 3c, d). However, antigen positive cells were rarely detected in the lungs of infected CBA mice and their concentration was significantly lower than in BALB/c lungs 10 days p.i. ($p \leqslant 0.0005$, Table 3).

Liver

Infected BALB/c mice developed a severe focal hepatitis which began 7 days p.i. with a few scattered foci of hepatocytic necrosis, nuclear inclusion-bearing hepatocytes and aggregates of granulocytes, macrophages and lymphocytes. By 10 days p.i. these foci were larger and more numerous than at earlier periods and many necrotic cells and hepatocytes with intranuclear inclusions were found. Such cells were shown to contain MCMV antigen. A few small foci of necrotic cells surrounded by granulocytes and macrophages were seen in the livers of CBA mice 10 days p.i. Prior to that lesions could not be detected.

Spleen

In infected BALB/c mice 4 days p.i., the concentration of megakaryocytes in the spleen was reduced and the red pulp was congested. Some of the mononuclear cells in the red pulp were enlarged and contained intranuclear amphophilic inclusions. These inclusion-bearing cells were still present 10 days p.i., together with a few necrotic cells and mild infiltration of granulocytes. The megakaryocytic concentration was similar to that of uninfected controls by day 10. The nuclei of large mononuclear cells in the red pulp of infected BALB/c mice were found to contain MCMV antigen. Apart from an occasional necrotic cell, few changes were seen in the spleens of CBA mice at the periods of sampling.

Discussion

The results of this study have shown the marked effect of host genotype on the severity of the disease induced by MCMV infection of newborn mice. BALB/c mice (H-2^d) had significantly higher MCMV titres, larger numbers of MCMV-antigen positive cells and more extensive tissue damage in all organs examined when compared to the CBA (H-2^k) strain. Severe disease in the BALB/c mice culminated in their death after day 10 p.i. while the majority of CBA mice survived. While retarded physical growth has been shown previously in MCMV-infected newborn mice [17], our study showed that this effect was also influenced by the host genotype with BALB/c mice being considerable more runted than CBA mice. Previous lethality studies using congenic adult and newborn mice differing only in their H-2 haplotype have established that resistance to MCMV is mainly controlled by genes of the H-2 complex, with mice having the H-2^k haplotype being relatively resistant compared to strains with other haplotypes [7, 26].

The involvement of the liver and spleen seen in the susceptible newborn BALB/c mice has been reported previously in adult mice and is not a unique feature of perinatal MCMV infection. A more striking aspect of this disease was the severe damage and high virus titres found in the lungs and hearts of BALB/c mice. BALB/c newborns developed severe pneumonitis coupled with high MCMV titres and relatively large numbers of MCMV-antigen-positive cells in their lungs. This severe pneumonitis observed after MCMV infection of newborn mice has not been previously observed to our knowledge.

Our observations in newborn BALB/c mice are in contrast to the findings in the lungs of i.p. inoculated adult mice where high virus titres and extensive pulmonary damage are not generally observed unless immunosuppressive regimes are used [6]. In one study, MCMV replication in the lungs of adult mice inoculated i.p. with MCMV was reported but the associated pneumonitis was only minimal [23]. After intranasal inoculation of adult mice, pneumonitis is not observed, despite the presence of replicating virus, unless the mice are immunosuppressed [24, 25]. Thus, newborn mice are more susceptible to developing MCMV pneumonitis than are their adult counterparts. Pneumonitis and MCMV replication in the lungs of immunosuppressed adult mice are also influenced by the host genotype and as in our study, BALB/c mice have been found to be relatively susceptible to MCMV lung infection compared to CBA mice [25]. As HCMV-induced pneumonitis also occurs predominantly in neonates and immunocompromised individuals, this emphasises the relevance of the murine model in the study of CMV-induced pneumonitis.

It has been proposed that immunosuppression-induced pneumonitis is immunologically mediated in adult mice [24]. As the newborn animal is immunologically immature, it is possible that much of the damage seen is a result of direct viral damage. This is supported by our finding of high MCMV titres in the lung and large numbers of MCMV-antigen-positive cells in areas of tissue

damage. In future studies, we propose to examine the nature of the cellular infiltrate and the mechanisms involved in the development of pneumonitis in newborn mice and to compare them with mechanisms operating in immuno-suppressed adult mice. By comparing our results to a recent study in adult mice (P. Price, pers. comm.), it would appear that the level of MCMV infection was greater and the numbers of inflammatory cells lower in the newborn lung than in the adult lung, although the composition of the adult and newborn inflammatory response appeared to be similar from these preliminary observations.

The susceptible BALB/c mice also developed severe endocarditis and myocarditis coincident with high MCMV titres in their hearts. Like the pneumonitis, this was a striking feature of the infection and probably contributed to the death of these mice. The involvement of the heart in MCMV-infected newborn mice has been reported previously [5, 13]. Our results have shown that MCMV infection of the heart is genetically controlled as relatively low MCMV titres and minor tissue damage were seen in CBA hearts compared to BALB/c hearts. Genetically controlled myocarditis has also been reported in MCMV-infected adult mice but the level of MCMV infection in the heart relative to other organs appeared to be much less in susceptible adult mice compared to newborn BALB/c mice [12]. MCMV invasion of the newborn heart appeared to begin with infection of the endocardial and subendocardial cells and then spread centrifugally. However, the factors that govern the severity of atrial infection were unclear. There have been infrequent reports of HCMV infection of the heart in perinatally infected infants [10, 22]. However, given the severity of the disease we observed and the similarities between the human and mouse model, it would be worthwhile to further investigate the level and distribution of cardiac involvement in HCMV-infected infants.

Also of interest was the occurrence of MCMV-infected cells in the endothelial and myointimal cells of the coronary arteries of BALB/c mice. Recently, several reports have suggested a role for HCMV in the etiology of atherosclerosis. HCMV nucleic acids have been demonstrated in the arteries of patients with and without atherosclerosis [9] and both nucleic acid and viral antigen have been demonstrated in cells cultured from atherosclerotic and normal vessels [14, 19]. However, sections of vessels did not reveal any HCMV antigen and infectious virus could not be isolated, suggesting that the vessels are a site of latency for HCMV [9, 14]. Experiments showing that atherosclerotic plaques can be induced in chickens by the herpesvirus Marek's disease virus support the theory for the viral etiology of atherosclerosis [15]. There has been one report of the occurrence of cytomegalic cells and HCMV antigens in vascular endothelial cells in an HCMV-infected infant [10]. Considering that atherosclerosis may have it origins in childhood [20], our finding that MCMV infects endothelial cells in newborn BALB/c mice may be of relevance in elucidating the role of viral infection in the induction of atherosclerosis and the influence of the host genotype on the incidence and severity of such lesions. In future studies, we propose to examine the consequences of sublethal MCMV infection of newborn mice on the development of atherosclerosis later in life.

The findings of this study suggest that the newborn mouse is a relevant model for perinatal HCMV infections, particularly in the study of HCMV pneumonitis in transfusion-infected infants. We also report that myocarditis is a major feature of newborn MCMV infection and should be further investigated as reflecting another possible manifestation of HCMV infection in premature infants. The finding of MCMV-infected cells in the coronary arteries of susceptible BALB/c mice may also have important implications in the pathogenesis of atherosclerosis. In addition, since MCMV-induced disease in newborn mice was genetically controlled, we propose that genetic factors may significantly influence the outcome of perinatal HCMV infections in man.

Acknowledgements

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