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Type I Interferons and IRF-1 Play a Critical Role in the Control of a Gammaherpesvirus Infection

B. M. Dutia,[1] D. J. Allen, H. Dyson, and A. A. Nash

Department of Veterinary Pathology, University of Edinburgh, Summerhall, Edinburgh, EH9 1QH, United Kingdom

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The murine gammaherpesvirus 68 (MHV-68) is an ideal model system for the study of interactions between gammaherpesviruses and their hosts. Intranasal infection of mice with MHV-68 results in replication of the virus in the lung epithelium followed by latent infection of B cells. Resolution of productive MHV-68 infection depends on the adaptive immune system, but little is known about the role of innate immune mechanisms and the early interaction between the host and the virus. In this report, we have used mice that are deficient in components of the early defence system, the common type I interferon (IFN) receptor (IFN R), the transcriptional activator IRF-1, and the inducible nitric oxide synthase, to investigate the contribution of these mechanisms to control of MHV-68 infection. We show that while wild-type mice are highly resistant to infection with MHV-68, mice unresponsive to type I IFNs (IFN-α/β $R^{-/-}$) are highly susceptible to the virus. At high multiplicities of infection (m.o.i; 4×10^6 PFU), 80–90% of IFN-α/β $R^{-/-}$ mice succumb to infection, and at low m.o.i. (4×10^3 PFU), 50% mortality rates occur. Both high and low doses of virus lead to 100- to 1000-fold higher lung virus titres in IFN-α/β $R^{-/-}$ mice than are found in wild-type mice and result in systemic dissemination of the virus. Latently infected cells are detectable in the spleens of IFN-α/β $R^{-/-}$ mice earlier than in wild-type mice, and the numbers of latently infected cells are 10-fold higher in the IFN-α/β $R^{-/-}$ mice during the acute phase of infection. We find IRF-1 has a critical role in protection from fatal disease, whereas inducible nitric oxide synthase does not appear to be important. The results indicate that innate immune mechanisms are critical for the early control of MHV-68 and may play a role in the establishment of latency.

INTRODUCTION

The gammaherpesviruses are an important group of pathogens in both humans and animals. *In vivo* studies on the human gammaherpesviruses, Epstein–Barr virus (EBV) and Kaposi's sarcoma herpesvirus (HHV-8), are severely limited and therefore murine gammaherpesvirus 68 (MHV-68; Blaskovic *et al.,* 1980; Efstathiou *et al.,* 1990) has been identified as an important model for investigating host responses to virus infection.

There is good evidence that the adaptive immune system plays a crucial role in MHV-68 infection. Intranasal infection with the virus results in acute replication in the lungs followed by latent infection in B lymphocytes (Sunil-Chandra *et al.,* 1992a,1992b). Clearance of the lung infection is initially dependent on $CD8^+$ T cells (Ethisham *et al.,* 1993), but evidence from studies in MHC class II-deficient mice suggests that in the longer term, $CD4^+$ T cells play a role in controlling the replication of virus (Cardin *et al.,* 1996). $CD4^+$ T cells are also responsible for the cell proliferation resulting in splenomegaly, the transient peak in the number of latently infected cells that occurs during the second week postinfection (p.i.), and the infectious mononucleosis-like syndrome that follows splenomegaly (Usherwood *et al.,* 1996; Cardin *et al.,* 1996; Tripp *et al.,* 1997).

Innate immune mechanisms are crucial for the control of virus infection before the recruitment of adaptive defence systems. The type I interferons (IFNs; IFN-α and IFN-β) play a vital role in the initiation of innate immunity through a variety of mechanisms, including the activation of natural killer (NK) cell cytotoxicity, regulation of cytokine and cytokine receptor expression, and early induction of the inducible nitric oxide synthase (iNOS) (reviewed in Biron, 1998; Diefenbach *et al.,* 1998). They also directly inhibit the replication of many viruses (Vilcek and Sen, 1996). Previous studies have shown that the type I IFNs and iNOS are important in the survival of mice infected with high doses of MHV-68 (Virgin *et al.,* 1997; Kulkarni *et al.,* 1997), but there is no published study on the kinetics of infection in the absence of these innate defences.

We studied MHV-68 infection in mice with a targeted disruption in an essential chain of the type I IFN receptor gene (IFN-α/β $R^{-/-}$; Muller *et al.,* 1994). We also investigated the role of the iNOS gene (Wei *et al.,* 1995) and the transcription factor IRF-1 (Matsuyama *et al.,* 1993) during MHV-68 infection. The IRF-1 gene is important, but not essential, for the induction of type I IFNs and for the upregulation of type I and type II IFN-inducible genes

[1] To whom reprint requests should be addressed. Fax: 44-131-650-6511. E-mail: B.M.Dutia@ed.ac.uk.

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(Miyamoto *et al.,* 1988; Pine *et al.,* 1990; Matsuyama *et al.,* 1993; Kimura *et al.,* 1994). IRF-1$^{-/-}$ mice are deficient in NK cell function (Duncan *et al.,* 1996) and in the ability to mount Th1 responses (Lohoff *et al.,* 1997). We show that IFN-α/β R$^{-/-}$ mice are highly susceptible to MHV-68 infection. The virus replicates to high titres, is widely disseminated, and causes death at doses that are sublethal in wild-type mice. IFN-α/β R$^{-/-}$ mice exhibit impaired clearance of the acute infection and altered kinetics of latent infection. Mice lacking the IRF-1 gene behave similarly to the IFN-α/β R$^{-/-}$ mice. However, iNOS is not essential for the control of MHV-68 infection.

RESULTS

Infection of IFN-α/β R$^{-/-}$ mice with MHV-68

To investigate the role of the type I IFNs in MHV-68 infection, groups of 10 wild-type 129Sv and 10 IFN-α/β R$^{-/-}$ mice were infected with various doses of MHV-68. Figure 1 shows the results of a representative experiment in which infection with 4×10^6 PFU of virus led to a 30% mortality rate in wild-type mice and a 90% mortality rate in the IFN-α/β R$^{-/-}$ mice. When wild-type mice were infected with $\leq 4 \times 10^5$ PFU, all the mice survived the infection, whereas 80% of the IFN-α/β R$^{-/-}$ died in response to 4×10^5 PFU and 50% died in response to 4×10^3 PFU. The survival time of the IFN-α/β R$^{-/-}$ mice was dependent on the multiplicity of infection. The mean time to death of 50% of the mice was 7–8 days at high doses of virus, whereas at 4×10^3 PFU it was 13 days. In some experiments, we found IFN-α/β R$^{-/-}$ mice infected with low doses of virus dying up to 3 weeks p.i. (data not shown). Interestingly, no deaths occurred in the wild-type mice until 11 days p.i. These data show that type I IFNs are important in the control of MHV-68 infection. The results also suggest that type I IFNs may continue to play a role at times when adaptive immune mechanisms have become activated.

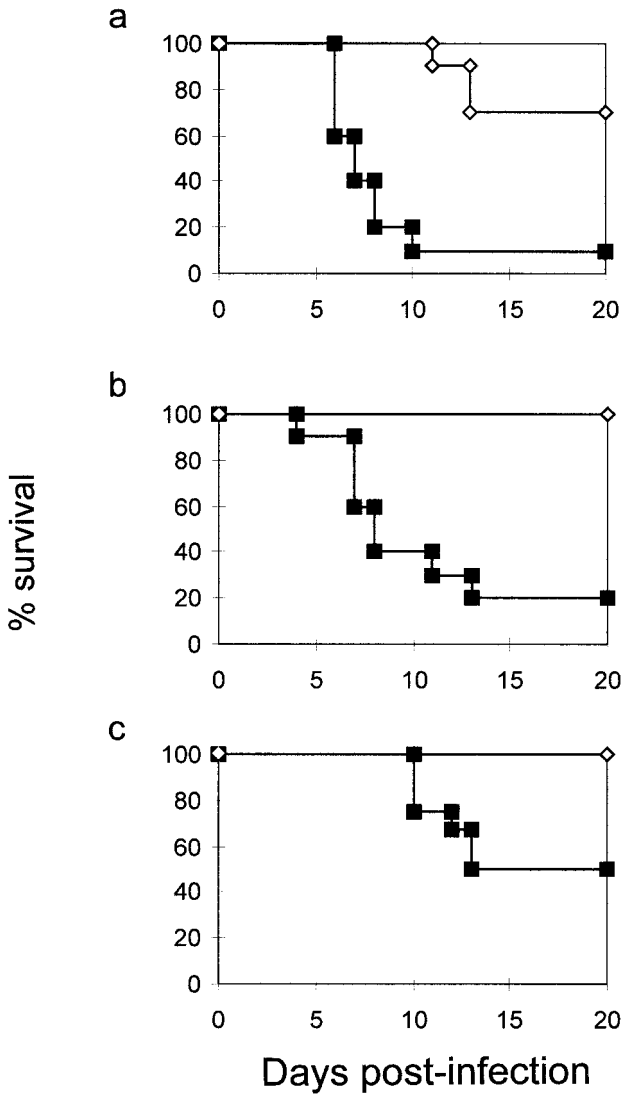

FIG. 1. Survival rates of wild-type and IFN-α/β R$^{-/-}$ mice infected intranasally with MHV-68. Groups of 10 mice were infected with various doses of virus and monitored daily. Results are representative of two experiments. (a) 4×10^6 PFU/mouse. (b) 4×10^5 PFU/mouse. (c) 4×10^3 PFU/mouse. ◇, Wild-type 129Sv mice. ■, IFN-α/β R$^{-/-}$ mice.

Infectious virus replication in IFN-α/β R$^{-/-}$ mice

The replication of MHV-68 in IFN-α/β R$^{-/-}$ mice was investigated by determining the titres of infectious virus present in the lungs and adrenal glands of wild-type and IFN-α/β R$^{-/-}$ mice. Age- and sex-matched mice, four per time point, were infected with high (4×10^5 PFU/mouse) or low (4×10^3 PFU/mouse) doses of virus. Figure 2 shows the lung virus titres obtained in a representative experiment. At both high and low doses of virus, the peak titres of virus in the lungs of IFN-α/β R$^{-/-}$ mice were 10^7 to 10^8 PFU/lung. This is 100- to 1000-fold higher than that in wild-type mice. The peak lung titres of virus were independent of the infectious dose that the mice received, although the peak titres occurred 2 days later at lower infectious doses. In IFN-α/β R$^{-/-}$ mice infected with 4×10^3 PFU virus, infectious virus could be recovered from the lungs for up to 3 weeks p.i., indicating impaired clearance. When mice were infected with 4×10^5 PFU virus, no virus was recovered at day 12 or later. However, because 90% of mice infected with this dose die within 10 days, the mice sampled at day 12 or later represent the 10% that have survived the infection and recovered.

The adrenal gland is readily infected by MHV-68, and we use this tissue as an indicator of systemic spread. In IFN-α/β R$^{-/-}$ mice, infectious virus was readily detected in the adrenal glands. Similar titres were found in mice infected with high and low doses (Fig. 3) and, as with the lungs, there was a lag in the appearance of peak virus titres in the mice infected with 4×10^3 PFU. All mice infected with high doses of virus had cleared virus from

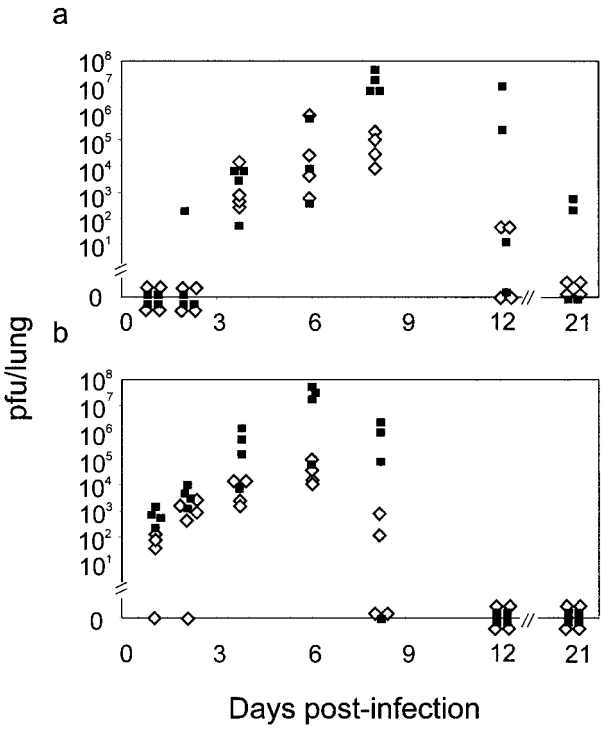

FIG. 2. Lung infectious virus titres in mice infected with MHV-68. Points represent individual mice. (a) Mice infected with 4×10^3 PFU virus. (b) Mice infected with 4×10^5 PFU virus. ◇, Wild-type 129Sv mice. ■, IFN-α/β $R^{-/-}$ mice. The limit of detection was 10 PFU/lung.

this tissue by day 12 p.i. Similarly, the low-dose mice had cleared the adrenal gland infection by 12 days, although virus could still be detected in the lungs of this group at 21 days p.i. No infectious virus could be detected in the adrenal glands of wild-type mice at any time after infection.

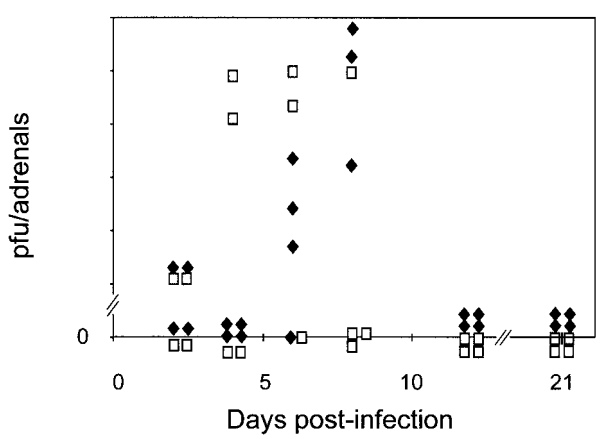

FIG. 3. Replication of MHV-68 in the adrenal glands of IFN-α/β $R^{-/-}$ mice. Points represent individual mice. ◆, Mice infected with 4×10^3 PFU virus. □, Mice infected with 4×10^5 PFU virus. The limit of detection was 10 PFU/adrenal. No infectious virus could be detected in the adrenal glands of wild-type mice.

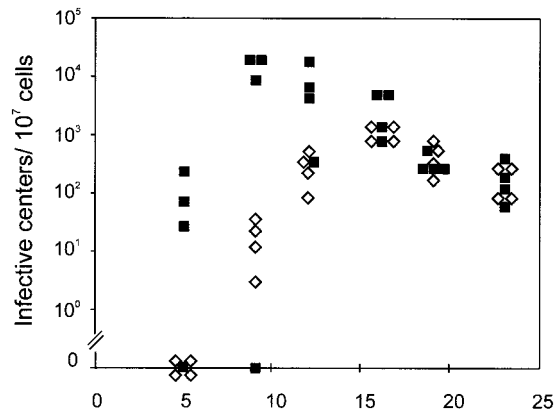

FIG. 4. Infective centers in the spleens of mice infected with 4×10^3 PFU MHV-68. Points represent individual mice. ◇, Wild-type 129Sv mice. ■, IFN-α/β $R^{-/-}$ mice. The limit of detection was 1 infective center/spleen.

Latent virus infection in IFN-α/β $R^{-/-}$ mice

We investigated the appearance of latently infected cells in the spleens of wild-type and IFN-α/β $R^{-/-}$ after infection with 4×10^3 PFU of virus. The lower multiplicity was chosen because of the low survival rates of IFN-α/β $R^{-/-}$ mice infected at higher doses. Figure 4 shows a representative experiment in which latent virus could be detected in the spleens of IFN-α/β $R^{-/-}$ mice as early as 5 days p.i. In contrast, no latently infected cells were detected in wild-type mice until 9 days p.i. The early appearance of latently infected cells in the spleen did not correlate with infectious virus in the lung where there was no difference between wild-type and IFN-α/β $R^{-/-}$ mice at early times (Fig. 2). The peak numbers of latently infected cells were 10-fold higher than those found in wild-type mice and occurred 3–6 days earlier in IFN-α/β $R^{-/-}$ mice. By 3 weeks p.i., however, similar numbers of latently infected cells were found in both groups of mice. Unlike wild-type mice, infectious virus could be detected in the spleens of IFN-α/β $R^{-/-}$ mice (data not shown). However, the levels were no more than 10–100 PFU/spleen, and this was taken into account when determining the latent virus titres shown in Fig. 4.

Infection in IRF-$1^{-/-}$ and $iNOS^{-/-}$ mice

IRF-$1^{-/-}$ mice were highly susceptible to MHV-68 infection and behaved similarly to the IFN-α/β $R^{-/-}$ mice. All mice exhibited clinical symptoms, and 70% of mice died in response to 4×10^5 PFU (Fig. 5).

In contrast, iNOS-deficient mice were resistant to infection with 4×10^5 PFU MHV-68. There was no sign of clinical disease and no deaths by day 17. These mice cleared the lung infection by day 7 and developed splenomegaly and persistent infection in the spleen with the same kinetics as wild-type 129 mice (data not shown).

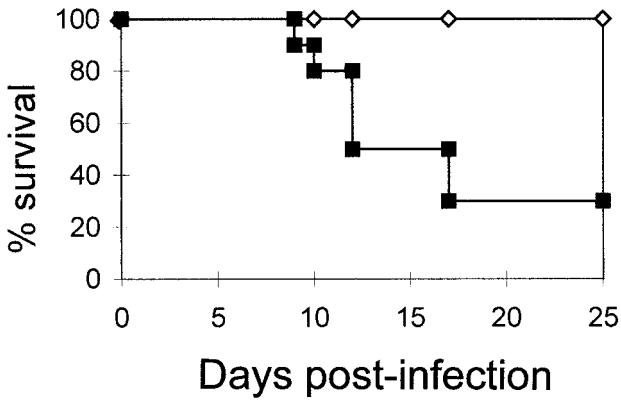

FIG. 5. Survival rates of wild-type and IRF-1 $R^{-/-}$ mice infected intranasally with 4×10^5 PFU MHV-68. Ten wild-type and 10 IRF-1 $R^{-/-}$ mice were infected. Results are representative of two experiments. ◇, Wild-type mice. ■, IRF-1 $R^{-/-}$ mice.

Histological analysis of infection in IFN-α/β $R^{-/-}$ mice

Histological examination of haematoxylin and eosin-stained sections of lung showed that infection of both wild-type and IFN-α/β $R^{-/-}$ mice with high or low doses of MHV-68 resulted in a general pattern of interstitial pneumonia with perivascular cuffing. However, at both doses of virus IFN-α/β $R^{-/-}$ mice showed severe pathological changes including signs of focal haemorrhage and patchy necrosis in the lungs (data not shown). Figure 6 shows haematoxylin and eosin-stained sections of lung from wild-type (Fig. 6a) and IFN-α/β $R^{-/-}$ (Fig. 6c) mice 8 days after infection with 4×10^5 PFU MHV-68 together with serial sections stained with polyclonal antiserum to MHV-68 lytic cycle antigens (Figs. 6b and 6d). Considerable lymphocytic infiltration can be seen in the wild-type lung, but only limited numbers of virus-positive cells are present. In contrast, the IFN-α/β $R^{-/-}$ lung shows extensive spread of viral antigen. Viral replication in peripheral tissues was also examined by antibody staining. In wild-type mice, an occasional cell containing replicating virus could be detected in the spleen (Fig. 6e). In IFN-α/β $R^{-/-}$ mice, large numbers of positive cells were scattered throughout the red and white pulp. Antigen-positive cells were also found in the liver of IFN-α/β $R^{-/-}$ mice (data not shown). These results

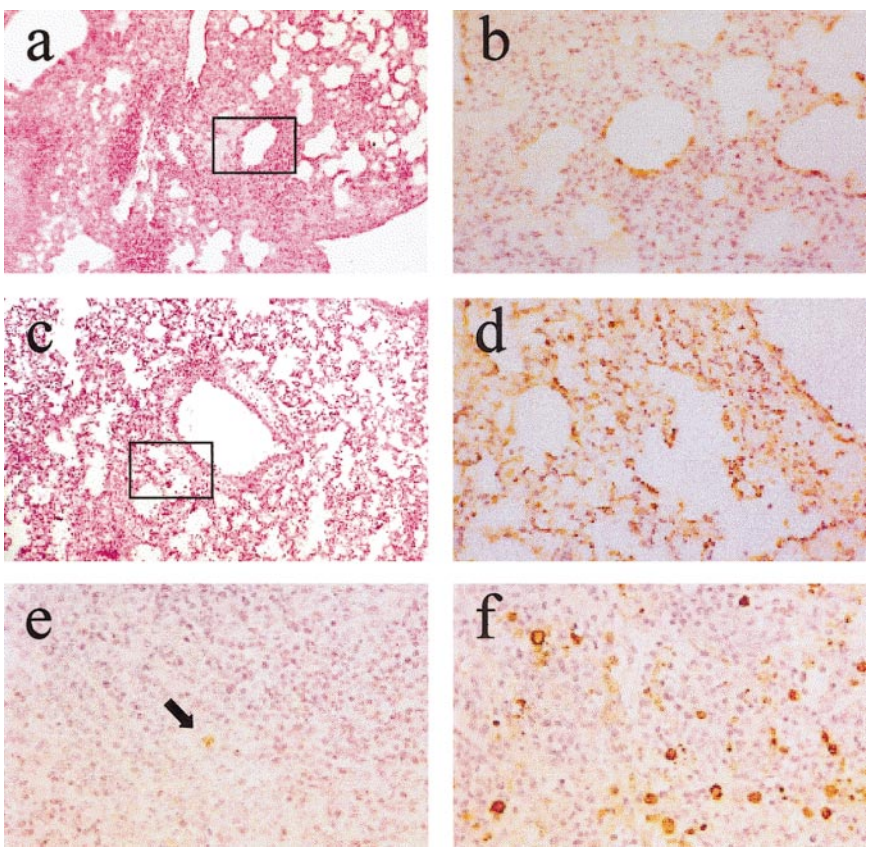

FIG. 6. Histochemical staining of tissues from wild-type and IFN-α/β $R^{-/-}$ mice. Sections of lung and spleen 8 days after infection with 4×10^5 PFU were stained with haematoxylin and eosin or with polyclonal rabbit anti-MHV-68. MHV-68 positive cells were stained brown with diaminobenzidine. (a) Wild-type lung, haematoxylin and eosin (×80). (b) Wild-type lung, anti-MHV-68 (×200). (c) IFN-α/β $R^{-/-}$ lung, haematoxylin and eosin (×80). (d) IFN-α/β $R^{-/-}$ anti-MHV-68 (×200). (e) Wild-type spleen, anti-MHV-68 (arrow indicates positive cell; ×320). (f) IFN-α/β $R^{-/-}$ spleen, anti-MHV-68 (×320). Boxes in a and c indicate areas enlarged in b and d.

illustrate the importance of the type I IFNs in the control of MHV-68 infection. Histological examination of lung tissue from IRF-1$^{-/-}$-infected mice showed severe pathological changes similar to those seen in IFN-α/β R$^{-/-}$ mice (data not shown).

DISCUSSION

IFN-α/β R$^{-/-}$ mice have been used to investigate the role of type I IFNs in a number of virus infections, including the RNA viruses lymphocytic choriomenigitis virus, vesicular stomatitis virus (VSV), Semliki forest virus, and Theiler's virus and the DNA virus vaccinia virus (Van den Broek *et al.,* 1995). In all of these experiments, type I IFNs were shown to play a major role in the control of infection. Evidence for a role of type I IFNs in herpesvirus infections comes from studies on the administration IFNs (Rasmussen and Farley, 1975; Fish *et al.,* 1983) and from work that demonstrates the importance of NK cells in clearance of infection (reviewed in Biron, 1997). Studies with EBV indicate that type I IFNs can downregulate proliferative responses to EBV *in vitro* (Aman and von Gabain, 1990), and evidence that the EBERs interfere with the function of IFN *in vitro* (Clarke *et al.,* 1991) suggests that type I IFN activity is important in EBV infection. The data presented here provide the first detailed study of the consequences of a lack of type I IFN activity in an acute gammaherpesvirus infection and in the establishment of latent infection.

IFN-α/β R$^{-/-}$ mice infected at high doses died quickly in response to MHV-68 infection. At 6 days p.i., lung virus titres were 100- to 1000-fold higher than those in the wild-type mice. However, some mice survived the infection and by day 12 p.i. had cleared all virus from their lungs. This is unlikely to be due to poor infection because 50% of mice infected with low doses had not cleared virus by this time. The kinetics of the infection in mice infected at low doses differed from those in the high-dose mice. The low-dose mice had a longer mean survival time and higher survival rate even though the lung titres were similar to those in mice infected at high doses. Interestingly, infectious virus could be recovered from the lungs of these mice at 21 days p.i. It is likely that the higher survival rate of the low-dose mice reflects the activation of protective cytotoxic T lymphocyte and antibody responses in the second week of infection. This does not, however, account for the slow clearance of the virus from the lungs. Type I IFNs have long been known to activate NK cells (Biron, 1997) and recently have been shown to play a role in activation of memory T cells (Tough *et al.,* 1996). The results presented here raise the question as to whether they also have a role in the activation of primary CD8$^+$ T cell responses similar to the activation of NK cells. This is currently under investigation.

Infectious virus assays indicated that in IFN-α/β R$^{-/-}$ mice virus replication occurred in tissues other than the lungs. Such replication was not detected in wild-type 129 mice by infectious virus assays, although occasional virus-positive cells were detected by immunohistochemical staining. Large numbers of positive cells were detected by antibody staining in the IFN-α/β R$^{-/-}$ mice. Antibody staining does not distinguish between productive and abortive infections, but it is likely that in the mutant mice staining detects replicating virus. Replicating virus has been found in the adrenal glands of young BALB/c mice (Sunil-Chandra *et al.,* 1992a) and in mice depleted of CD8$^+$ T cells (Ethisham *et al.,* 1993). The spread of virus from lungs to spleen requires B cells (Usherwood *et al.,* 1996), and it is likely that dissemination of infection occurs via blood-borne B cell-associated virus. In very young or CD8$^+$ T cell-depleted mice, virus may spread from B cells in the absence of a competent immune system. This may also be the case in IFN-α/β R$^{-/-}$ mice. However, an alternative explanation may be that lack of type I IFN allows latently infected B cells to readily undergo a productive infection, resulting in peripheral spread of the virus.

Higher numbers of latently infected cells were found 14 days p.i. in the spleens of IFN-α/β R$^{-/-}$ mice compared with wild-type. It is interesting that latently infected cells appeared in the spleens of IFN-α/β R$^{-/-}$ mice earlier than in wild-type mice even though virus titres in the lung were identical until after day 6. Exactly how this might occur is unclear, but it is likely that type I IFNs have a role in preventing infection of B cells. Hence cells are infected at earlier time points and in greater numbers in IFN-α/β R$^{-/-}$ mice. At later times, however, the IFN-α/β R$^{-/-}$ mice have similar numbers of latently infected cells to those found in wild-type mice. This parallels the situation in IFN-γ R$^{-/-}$ mice, which have peak latency loads 100- to 1000-fold higher than those in wild-type mice but establish similar latency loads in the longer term (Dutia *et al.,* 1997). Similarly, the long-term latency levels in CD4$^+$ T cell-depleted mice are the same as in undepleted mice, although these mice behave differently during the initial phase of infection (Nash *et al.,* 1996). The mechanism controlling the number of latently infected cells is not well understood, but CD8$^+$ T cells clearly play a role (Cardin *et al.,* 1996). It is likely that as the adaptive immune response develops, it is able to operate the same latency control mechanism in immunocompromised mice, such as the IFN-α/β R$^{-/-}$ mice and the IFN-γ R$^{-/-}$ mice, as in intact mice. These mechanisms are intriguing and require further study.

IRF-1$^{-/-}$ mice, unlike IFN-α/β R$^{-/-}$ mice, are resistant to infection with VSV, indicating that induction of a type I IFN-dependent antiviral state against VSV is independent of IRF-1 (Matsuyama *et al.,* 1993; Reis *et al.,* 1994; Muller *et al.,* 1994). Clearly, this is not the case for MHV-68. Induction of type I IFNs or in upregulation of activities such as the IFN-induced, double-stranded

RNA-activated protein kinase or 2-5A synthetase can occur in the absence of IRF-1 (Kimura *et al.,* 1994). Although these activities may be important in MHV-68 infection, the requirement for IRF-1 indicates that IRF-1-dependent activities, such as induction of NK cell activity, are an important component of the type I IFN-dependent antiviral state in MHV-68 infection.

Our results showing that a lack of iNOS has no effect on the replication of MHV-68 are in direct contrast to those of Kulkarni *et al.* (1997). This is not readily explained, but the mice used in this study were derived independently from those used by Kulkarni and coworkers, and the results may be attributable to the different backgrounds of the mice.

Type I IFN receptors are present on all cell types, including epithelial cells and lymphocytes (Aguet and Mogensen, 1983). The current experiments cannot differentiate between direct antiviral effects that occur in lung epithelial cells and indirect mechanisms such as activation of NK cytotoxicity. Experiments aimed at identifying the cell types involved in the protective effect are under way.

The type I and type II IFNs display similar antiviral activities and share signaling pathway components (Bach *et al.,* 1997). However, a comparison of MHV-68 infection in type I and type II IFN receptor-deficient mice indicates very different roles for the two types of IFN in MHV-68 infection. The lack of the IFN-γ receptor (IFN-γ $R^{-/-}$) has no effect on the clearance of MHV-68 from the lungs or, in our experience, on the survival of the mice. MHV-68-infected IFN-γ $R^{-/-}$ mice, however, develop severe pathological and virological changes in the spleens (Dutia *et al.,* 1997). As shown here, IFN-α/β $R^{-/-}$ mice are highly susceptible to MHV-68 infection, but there is no evidence of splenic atrophy and splenomegaly occurs as in wild-type mice. Although the mechanism of splenic atrophy is presently unknown, it does not appear to involve common pathways with those involved in type I IFN activity.

Type I IFNs have been shown to induce bystander T cell proliferation of $CD8^+$ T cells with a memory phenotype (Tough *et al.,* 1996) and thus have a potential role in the generation of immunological memory. We are currently investigating the frequency of T cell memory in wild-type and IFN-α/β $R^{-/-}$ mice and monitoring the frequency of spontaneous reactivation in these mice as a correlate of a diminishing immunological control.

MATERIALS AND METHODS

Virus

MHV-68 was originally obtained from Prof. Blaskovic (Blaskovic *et al.,* 1980). Working stocks of MHV-68 were prepared by infection of BHK-21 cells with MHV-68 clone g2.4 (Efstathiou *et al.,* 1990) at low multiplicity (0.1 PFU/cell) as previously described (Sunil-Chandra *et al.,* 1992a).

Mice

Inbred wild-type 129/Sv and IFN-α/β $R^{-/-}$ 129/Sv mice (Muller *et al.,* 1994) were obtained from B & K Universal Limited (Hull, U.K.) and maintained as a closed colony. IRF-$1^{-/-}$ mice (Matsuyama *et al.,* 1993) were obtained from the Department of Medical Microbiology, University of Edinburgh. $iNOS^{-/-}$ mice were a generous gift from Prof. F. Y. Liew, University of Glasgow. Age- and sex-matched mice were infected under halothane anaesthesia at 6–12 weeks of age.

Virus assays

Virus stocks were assayed on BHK-21 cell monolayers (Sunil-Chandra *et al.,* 1992a). To determine virus titres in infected mice, various tissues were frozen at −70°C, thawed and homogenised, and then refrozen at −70°C. After thawing, the homogenates were centrifuged to remove particulate matter and titred on BHK-21 cell monolayers as described above for virus stocks. Latent virus was detected by an infective center assay (Sunil-Chandra *et al.,* 1992a). Briefly, spleen cell suspensions were prepared by teasing cells out of the capsule, red blood cells were lysed by brief resuspension in water, and the lymphocytes were cocultivated with BHK-21 cells for 5 days. Cell monolayers were fixed and stained, and the numbers of splenocytes giving rise to plaques counted. To detect infectious virus in splenocytes, these cells were frozen at −70°C, thawed, clarified by centrifugation, and assayed on BHK-21 cell monolayers.

Histological analysis

Tissues were fixed in 10% formol saline and processed to 5-μm paraffin wax-embedded sections. MHV-68 lytic cycle antigens were detected with polyclonal rabbit antiserum raised against infected cell lysate. Sections were blocked overnight with normal goat serum and then incubated for 2 h with polyclonal serum followed by biotinylated anti-rabbit Ig, streptavidin peroxidase (Boehringer Mannheim UK), and diaminobenzidine (Sigma). Serial sections were stained with haematoxylin and eosin and examined by light microscopy.

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