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The Mouse Cytomegalovirus Immediate-Early 1 Gene Is Not Required for Establishment of Latency or for Reactivation in the Lungs[▽]

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The immediate-early protein IE1 of human and mouse cytomegalovirus (MCMV) is one of the first proteins expressed during the productive infection cycle and upon reactivation from latency. The CMV IE1 proteins have been found to inhibit histone deacetylases, suggesting a role in the epigenetic regulation of viral gene expression. Consequently, the IE1 protein is considered to have a profound effect on reactivation, because small amounts of IE1 may be decisive for the switch to lytic replication. Here we asked if an MCMV Δie1 mutant is able both to establish latency and to reactivate from the lungs of latently infected mice. Since the Δie1 mutant was known to be attenuated during acute infection, we first defined conditions that led to comparable levels of viral genomes during latent infection with mutant and wild-type (wt) MCMV. Viral genome copy numbers dropped considerably at the onset of the latent infection but then remained steady for both viruses even after several months. Reactivation of the Δie1 mutant and of wt MCMV from latency occurred with similar incidences in lung explant cultures at 4, 7, and 12 months postinfection. The increase in the frequency of a subset of MCMV-specific memory T cells, a possible indicator of frequent transcriptional reactivation events during latency, was in a comparable range for both viruses. Recurrence of the Δie1 virus infection in vivo could also be induced by hematoablative treatment of latently infected mice. We conclude that the ie1 gene is not essential for the establishment of latency or for the reactivation of MCMV.

Infection with cytomegalovirus (CMV) is relatively common in humans but is rarely diagnosed, because the primary infection is usually asymptomatic or presents with only mild symptoms (35). After clearance of the primary infection by the immune system, a latent infection characterized by the absence of detectable infectious virus is established in infected individuals. The latent virus genomes persist in certain cell types, such as monocytes or endothelial cells, in many organs of the host (reviewed in references 21 and 47). The CMV infection may occasionally recur from this state of dormancy, but usually it remains undetected in healthy individuals, because the infection is rapidly controlled again by the immune system. Cellular immunity, in particular the cytotoxic T-cell response, has been recognized as crucial in the control of CMV replication and disease as well as for the prevention of reactivation (9, 40, 42). In immunocompromised patients, such as solid-organ or bone marrow transplant recipients, reactivation from latency can lead to high levels of CMV replication due to insufficient immune control (reviewed in reference 5). CMV recurrence in these patients is often associated with life-threatening disease; one severe complication is interstitial pneumonia, which is hard to control by medication and can be fatal.

The viral and cellular mechanisms that regulate the maintenance of the viral genome during latency and those that trigger reactivation are not well understood (for a review, see reference 53). In latently infected monocytes, the CMV genome has been found as an episome (4), in accordance with the latent state of other herpesviruses. CMV gene expression during latency is severely restricted; a few transcripts have recently been detected (3, 12, 22), but their significance is not yet known. It is generally believed that reactivation is initiated by transcriptional activation of the CMV major immediate-early (IE) genes (53). Various stimuli can activate cellular transcription factors, which then bind to the major immediate-early promoter just upstream of the coding region for the IE proteins. Studies with mouse CMV (MCMV), a widely used model for the analysis of CMV latency and reactivation (reviewed in references 19, 44, and 45), have indicated that cytokines associated with infections or allorejection are such stimuli (8, 20, 50). Interestingly, the signaling events triggered by cytokines do not necessarily result in the recurrence of MCMV infection; usually, they lead only to the synthesis of major IE transcripts, suggesting that additional processes are required (for instance, differentiation of the latently infected cells into a permissive state). Stochastic transcription events resulting in the generation of MCMV ie1 and ie2 transcripts have also been observed during latency (14, 27).

The transcription units encoding the major IE proteins of MCMV and human CMV (HCMV) display remarkable gene structure similarity (7, 32). The primary transcript is differentially spliced to generate two predominant mRNAs. One mRNA consists of exons 1 to 4, and the other is composed of exons 1 to 3 and 5. In HCMV, these mRNAs give rise to the IE1 p72 and IE2 p86 proteins, while the corresponding IE mRNAs of MCMV encode the 89-kDa IE1 phosphoprotein

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(25) and the 88-kDa IE3 protein (33). HCMV IE2 and MCMV IE3 serve as transactivator proteins, which are required for the transcriptional activation of viral genes expressed later in the productive infection cycle. The CMV IE1 proteins were initially characterized as regulatory coactivator proteins that support the transactivating function of the IE2 or IE3 protein (52). One prominent role that was reported for the IE1 proteins of both CMVs is the disruption of subnuclear structures called promyelocytic leukemia protein-associated nuclear bodies (PML NBs), or ND10 domains (1, 26, 63). These structures are characterized by the accumulation of several cellular proteins, such as Sp100, hDaxx, and PML, the latter being required for the formation of PML NBs. They are believed to play a role in the intrinsic antiviral defense of cells, since viral genomes entering the nuclei of infected cells are localized and possibly sequestered at or next to PML NBs (reviewed in reference 30). The antiviral function may be mediated via the PML protein (57, 58), which represses transcription by interacting with histone deacetylases (64), or via the Sp100 protein, which binds to the heterochromatin protein HP1, involved in transcriptional silencing (29). Thus, the IE1 protein seems to be required by the virus to counteract the antiviral function of PML NBs, thereby supporting the initiation of the productive infection cycle.

A direct interaction between the MCMV IE1 protein and the histone deacetylase HDAC2 has been described. This interaction results in reduced histone deacetylase activity (55), possibly facilitating the formation of an open chromatin structure of the viral genome. Similarly, an interaction between HDAC3 and the HCMV IE1 protein has been found (37). Consistent with an activity of the IE1 protein in epigenetic regulation of CMV gene expression, HCMV ie1 deletion mutants display a severe growth defect at a low multiplicity of infection (MOI) in cell culture (13, 34). In contrast to the HCMV mutant, an MCMV Δie1 deletion mutant grew with kinetics comparable to those of wild-type (wt) MCMV in various cell types in vitro, independently of whether infection was performed at a low or a high MOI (10). In vivo, the Δie1 mutant displayed an attenuated phenotype (10); the doubling time of the viral titers was significantly reduced in various organs, including the lungs (62).

Although an important role for the IE1 protein during latent infection or in reactivation has frequently been suggested, this concept has not been experimentally tested. In this study, we asked whether the MCMV ie1 gene plays a role in regulating the establishment of latency, in the maintenance of the latent genomes, or in the reactivation of MCMV from the lungs of latently infected mice. Unexpectedly, we found that a Δie1 mutant is able to reactivate from latency in explanted lung tissue in vitro as well as in vivo with an incidence similar to that for wt MCMV.

MATERIALS AND METHODS

Viruses. The generation of the bacterial artificial chromosome-derived MCMV strain MW97.01 (59) and of the Δie1 mutant (10) has been described previously. Strain MW97.01 has been shown to be as virulent as the MCMV Smith strain (59) and was designated wt MCMV in this study. The viruses were propagated on primary mouse embryonic fibroblasts (MEF), which were prepared from the embryos of pregnant BALB/c mice on day 17 of gestation. Virus stocks were produced as described previously (6).

MCMV infection of mice. Newborn female BALB/c mice (Charles River, Sulzfeld, Germany) were intraperitoneally infected 4 days postnatum with different doses of the MCMV strains in 50 μl of phosphate-buffered saline. Mice were housed in ventilated cages in an area otherwise free of murine pathogens as revealed by microbiological monitoring according to FELASA recommendations (38). At designated times after infection, mice were sacrificed, and organs were excised and homogenized by passing through a 0.55-mm wire mesh (Roth, Karlsruhe, Germany) prior to further processing. Animal experiments were approved under permission numbers 33-42502-05/1058 and 33.9-42502-04-07/1336 according to German federal law.

Quantification of infectious virus. Virus titers were determined by a plaque assay performed on subconfluent second-passage MEF monolayers in triplicate. Centrifugal enhancement at $500 \times g$ was applied for 30 min, followed by 2 h of incubation at 37°C. The inocula were then replaced by 500 μl of 0.75% (wt/vol) carboxymethylcellulose (Sigma-Aldrich, Schnelldorf, Germany) in growth medium per well. On day 5 postinfection (p.i.), plaques were counted, and viral titers per whole organ were calculated.

Quantification of viral genomes. Ten percent of the lung homogenates was used to extract genomic DNA (DNeasy blood and tissue kit; Qiagen), and 100 ng of extracted DNA was subjected to quantitative real-time PCR, performed as described previously (50). Plasmid pDrive_gB_PTHrP_Tdy was used to generate a standard curve in order to calculate the ratio of viral to mouse genomic DNA. PCR was performed in a final volume of 25 μl of Brilliant SYBR green mix (Stratagene, Amsterdam, The Netherlands) on an Mx3000P cycler (Stratagene).

Quantification of MCMV-specific T cells. Splenocytes were isolated from latently infected BALB/c mice. P815 mastocytoma cells (ATCC TIB-64) were pulsed for 2 h at 37°C with synthetic peptides 168-YPHFMPTNL-176 of the IE1 protein (16) and 257-AGPPRYSRI-265 of the m164 protein (17) (Eurogentec, Liege, Belgium) at 10^{-8} M, and then 10^5 splenocytes were coincubated with 10^5 of the peptide-loaded P815 cells for 20 h. Gamma interferon (IFN-γ)-producing cells were quantified by an enzyme-linked immunospot (ELISPOT) assay (eBioscience, San Diego, CA) according to the manufacturer's instructions. The Mann-Whitney statistical test (non-Gaussian) provided with the GraphPad Prism 5 program (GraphPad, La Jolla, CA) was used to compare the T-cell frequencies between different mouse groups. Differences were considered to be significant if the P value of the two-tailed test was <0.05.

Ex vivo reactivation assay. Lungs were isolated from latently infected BALB/c mice and cut into small pieces with scissors. The pieces from one lung were randomly distributed into the wells of a 6- or 12-well plate, and the cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. The medium was replaced twice a week, and supernatants were harvested over 6 weeks to monitor for the presence of infectious virus by transfer to MEF indicator cells. The cultures were analyzed microscopically for the occurrence of cytopathic effects 1 week posttransfer. NIH 3T3 cells infected with the recovered viruses were analyzed by immunoblotting using monoclonal antibodies Croma101 (anti-IE1 [αIE1]) and Croma103 (αE1) (both kindly provided by Stipan Jonjić, Rijeka, Croatia) and a secondary horseradish peroxidase-conjugated anti-mouse antibody (Dako, Hamburg, Germany). Signals were visualized with an ECL detection reagent (GE Healthcare, Munich, Germany) on a LAS3000 imager (Fuji, Düsseldorf, Germany).

In vivo reactivation. Hematoablative treatment of latently infected BALB/c mice was performed by total-body irradiation with a single sublethal dose of 6.5 Gy within 12 min using a Siemens Mevatron MX-2 system (Erlangen, Germany). For the following 8 days, mice were treated with the antibiotic cotrimoxazole (240 mg per liter of drinking water [Ratiopharm, Ulm, Germany]). Mice were sacrificed; the lungs were excised and homogenized; and the amounts of viral genomes and infectious virus were analyzed by PCR and plaque assay, respectively. The DNA of reactivated viruses was further analyzed by PCR using primers 5′-CCTCACTCTCAGAGGGCTTATGTAA-3′ and 5′-TTTAGAGAG ATGGAGCCCGC-3′, giving rise to a 2,465-bp product for wt MCMV and a 609-bp product for the Δie1 virus.

RESULTS

Growth of the Δie1 mutant in the lungs of infected mice and establishment of latency. Previous studies have shown that the lungs are a major site of MCMV latency and recurrence (2) and that infection of newborn mice results in a high viral genome load in the lungs (43). The viral load, in turn, defines the likelihood of the recurrence of MCMV infection (43). The newborn infection model seemed to us, therefore, to be highly
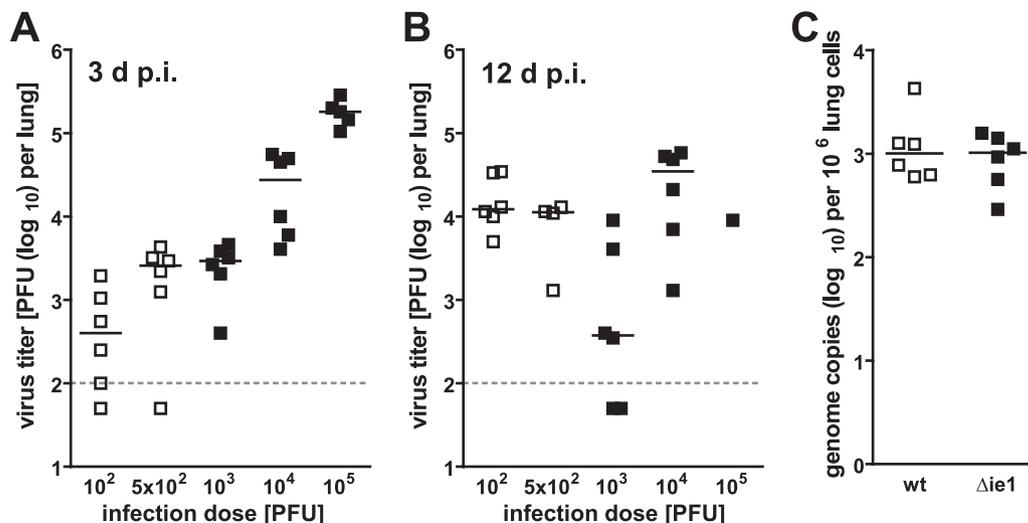
FIG. 1. Growth of the Δie1 mutant and viral loads established in the lungs of infected BALB/c mice. Groups of six female newborn BALB/c mice were intraperitoneally infected with the indicated doses of wt MCMV (□) or the Δie1 mutant (■). Viral titers in the lungs were quantified 3 (A) and 12 (B) days (d) p.i. Each data point represents the titer for an individual animal, and the horizontal bars indicate the medians for each group. The detection limit is indicated by the dashed lines. (C) Viral genome loads in the lungs of latently infected mice were measured 6 months after infection with 100 PFU of wt MCMV (□) or 1,000 PFU of the Δie1 mutant (■). The medians are indicated by horizontal bars.

suitable for evaluating the reactivation capacity of the Δie1 mutant in the lungs. Our first aim was to define infection conditions that resulted in similar multiplication of the Δie1 mutant and of wt MCMV in the lungs, because this was found to be a requirement for the establishment of a comparable viral genome load during latency (43). The Δie1 mutant was previously analyzed with adult mice, and attenuation was observed (10). Therefore, we first investigated the growth of the Δie1 mutant, in comparison to the growth of the wt virus, in the lungs of infected neonatal mice. Newborn BALB/c mice were infected intraperitoneally with different doses of the viruses, and virus titers in the lungs were measured at 3 and 12 days p.i. (Fig. 1). Based on previous data regarding the susceptibility of newborn mice to MCMV (15, 43) and the expected attenuation of the mutant (10), we used infection doses of 100 and 500 PFU of the wt virus and 10^3, 10^4, and 10^5 PFU of the Δie1 mutant. Infection with 500 PFU of the wt virus was lethal for two of six mice within 12 days, underlining the susceptibility of newborn mice to MCMV. At day 3 p.i., 10^3 PFU of the Δie1 mutant resulted in titers in the lungs comparable to those resulting from 500 PFU of wt MCMV (Fig. 1A), but on day 12 p.i., titers in Δie1-infected mice were >1 order of magnitude lower than those in mice infected with 100 or 500 PFU of wt MCMV (Fig. 1B). Infection with 10^4 PFU of the Δie1 mutant led to higher titers at both time points than the highest dose (500 PFU) tested for wt MCMV. At 10^5 PFU of the Δie1 virus, five of six mice succumbed to infection within 12 days. Since it was difficult to find inoculation doses that led to comparable titers of the two viruses in the lungs at both time points, we decided to start our experiments with a nonlethal dose of 100 PFU for the wt virus and with 10^3 PFU of the Δie1 mutant. This may result in higher titers for the mutant initially and in higher titers for the wt virus at later times. The intention, however, was to achieve similar loads of viral genomes during latency, since we considered this a precondition for comparing

the reactivation capacities of the mutant and the wt virus. Six months after infection with 100 PFU of wt MCMV or 1,000 PFU of Δie1, the loads of latent viral genomes in the lungs were determined by quantitative PCR. For both viruses, genome loads in the range of 10^3 viral genomes per 10^6 lung cells were found (Fig. 1C). In conclusion, we were able to establish infection conditions that resulted in similar loads of latent viral genomes in the lungs, thereby allowing us to study the role of the IE1 protein in latency and reactivation without any bias resulting from the attenuation of the Δie1 mutant in the acute infection phase.

Reactivation of the Δie1 mutant in lung explant cultures. Four months p.i., infectious virus was no longer detectable in the lungs of infected animals (see, e.g., Fig. 5B), indicating that a latent infection had been established. We chose lung explant cultures as described by Presti et al. (41) to study the reactivation of the Δie1 mutant. The recurrence of infectious MCMV in the cultures was monitored for 42 days. Starting at 2 weeks after explantation, infectious virus was found in the supernatants of the cultures. Altogether, the lung explant cultures from four of six mice infected with the Δie1 mutant and from five of six mice infected with wt MCMV gave rise to reactivated virus (Fig. 2A). In addition, we did not see an obvious difference in the kinetics of reactivation of the two viruses (data not shown). To verify that the viruses recovered from the lung explant cultures were identical to the viruses used for infection of the mice, the reactivated viruses were grown on fibroblasts, and the synthesis of the IE1 and E1 proteins was analyzed (Fig. 2B). These results demonstrated that the Δie1 mutant is able to establish latency and that the IE1 protein is dispensable for the reactivation of MCMV in explanted lung tissues.

Time course of the viral genome loads in the lungs. To analyze whether the IE1 protein may have a role in the maintenance of the viral genome load during extended periods of
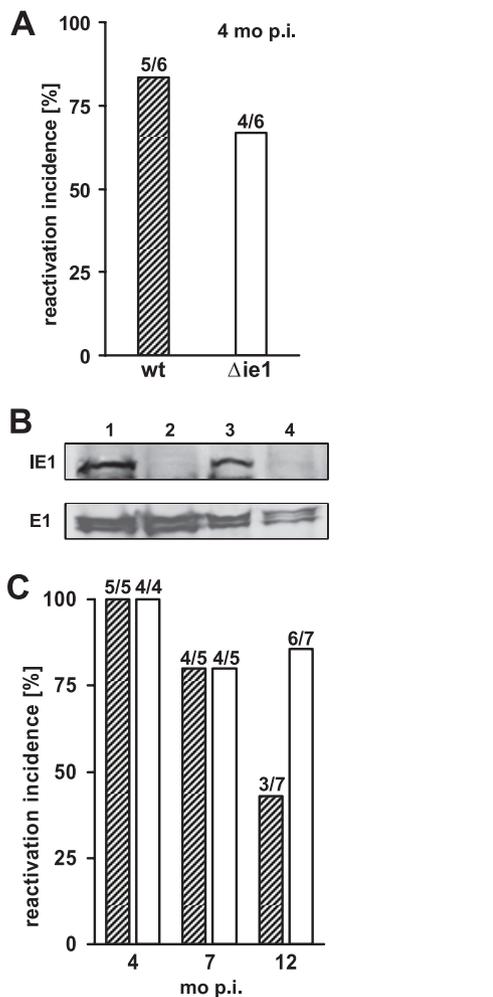
FIG. 2. Ex vivo reactivation of the Δie1 mutant. (A) Groups of six mice were infected with either 10^2 PFU of wt MCMV (hatched bar) or 10^3 PFU of the Δie1 mutant (open bar). Four months p.i., lungs of latently infected mice were explanted, and reactivation of the viruses in the explant cultures was monitored for 42 days. (B) The identities of the recovered viruses were verified by immunoblot analysis with monoclonal antibodies directed against the IE1 (top) or the E1 (m112/113) (bottom) protein. Lysates were obtained from cells infected with the viruses recovered from wt or Δie1 MCMV-infected mice (lanes 1 and 2, respectively) and, for comparison, from cells infected with wt or Δie1 MCMV (lanes 3 and 4, respectively). (C) Groups of four to seven mice were infected with either 10^2 PFU of wt MCMV (hatched bars) or 10^4 PFU of the Δie1 mutant (open bars). At 4, 7, and 12 months p.i., explant cultures of lung tissues were established, and the reactivation incidence was determined as described for panel A.
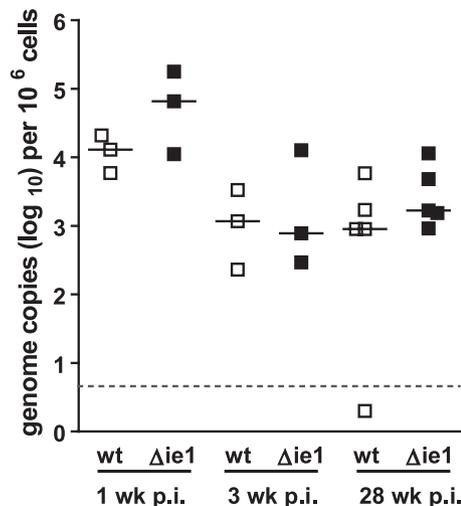

FIG. 3. Time course of viral genome loads in the lungs. Viral genome copy numbers were measured by quantitative PCR at the indicated time points after infection of newborn BALB/c mice with either 10^2 PFU of wt MCMV (□) or 10^4 PFU of the Δie1 mutant (■). The median values are represented by horizontal bars, and the detection limit is indicated by dashed lines.

latency, we infected newborn BALB/c mice and determined the genome loads in the lungs by quantitative PCR at different time points p.i. It is known that the viral genome copy numbers in the organs of infected mice drop considerably when the acute infection is cleared and the latent infection is established (11). In view of the sensitivity of the quantitative PCR and the need to detect a potential decline in the viral genome load during latency, we aimed to start with the maximal possible genome load at the onset of latent infection. To this end, we infected the mice with 10^4 PFU of the Δie1 mutant or 100 PFU of wt MCMV. Under these conditions, a slightly higher ge-

nome load was detected for the Δie1 mutant than for wt MCMV in the acute infection phase at 1 week p.i. (Fig. 3). During the following 2 weeks, the genome copy numbers decreased rapidly by approximately 1 (wt MCMV) or almost 2 (Δie1 mutant) orders of magnitude. Thereafter, however, the loads of both viruses were found to be maintained at this level until 28 weeks p.i. This finding showed that although there was a more pronounced reduction of genome copy numbers of the Δie1 mutant in the lungs around the time point when latency was established in the lungs, no further decline in the load of the Δie1 mutant in the subsequent latency phase was observed in comparison to the load of wt MCMV, indicating that the IE1 protein is not essential for the maintenance of the MCMV genome during latency.

Expansion of m164-specific T cells after infection with the Δie1 mutant. The majority of the MCMV-specific memory T cells in the chronic phase of infection are specific for only a few viral epitopes, and the frequency of these memory T cells increases over time (16, 17, 24, 36). While the mechanism driving this process, termed memory inflation, has not yet been clearly defined, a plausible explanation is the frequent and stochastic recurrence of viral gene expression during latency, leading to T-cell restimulation and expansion (reviewed in references 45 and 60). If the IE1 protein is involved in the opening of the viral chromatin structure or in the maintenance of the open structure, one might expect that the Δie1 mutant would switch on viral protein expression less frequently or to a lower extent and that therefore the accumulation of immunodominant memory T cells would be less prominent. Thus, we considered it worthwhile to measure the frequency of these MCMV-specific T cells at 7 and 12 months p.i. The proportion of T cells responding with IFN-γ production upon restimulation with the immunodominant m164 and IE1 peptides was determined by an ELISPOT assay (Fig. 4). Splenocytes of mice latently infected with wt MCMV responded to both peptides, while infection with the Δie1 mutant failed to

A

B
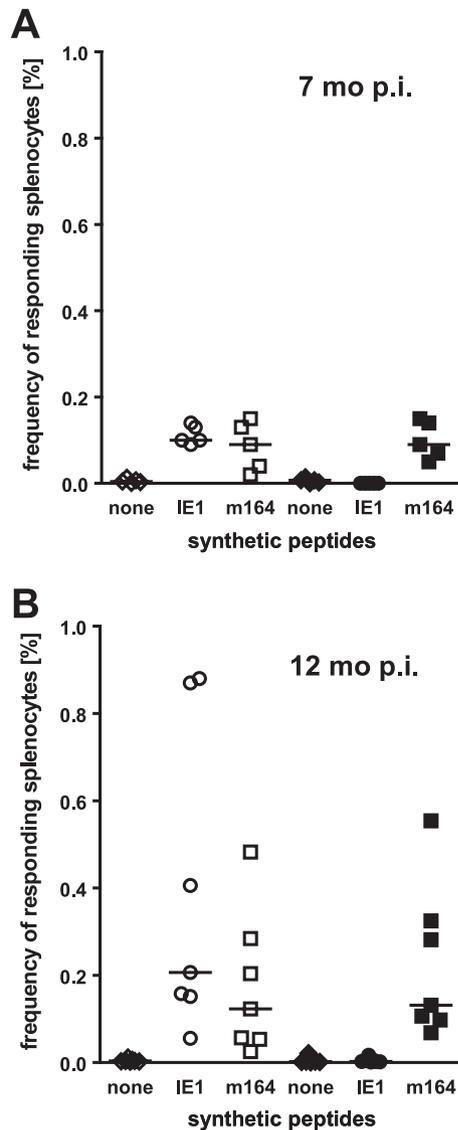

FIG. 4. Frequency of MCMV-specific T cells. Mice were infected with wt MCMV (open symbols) or the Δie1 mutant (filled symbols) as described in the legend to Fig. 3, and splenocytes were isolated 7 (A) and 12 (B) months p.i. IFN-γ-producing T cells were detected by an ELISPOT assay after restimulation with the immunodominant peptide of the IE1 (circles) or m164 (squares) protein or after treatment without peptides (diamonds). Medians are indicated by horizontal bars.

induce IE1-specific T cells, due to the absence of the IE1 protein (10). This sample, therefore, served as an additional control for the specificity of the assay. m164-specific T cells were present at the same frequency in mice infected with either of the viruses at 7 months p.i. (Fig. 4A). At 12 months p.i., the frequencies of these T cells showed larger differences between individual mice than at 7 months p.i., and the median frequency of the IE1-specific T cells in the wt MCMV-infected mice was doubled. The increases in the median frequencies of the m164-specific T cells were less prominent (1.37- and 1.46-fold) than the increase in the median frequency of the IE1-specific T cells. Most importantly, the frequencies of the m164-specific T cells were in a similar range in the Δie1

and wt virus-infected mice at 12 months p.i., and there was no statistically significant difference. We interpret our findings as a hint that transcriptional reactivation of viral protein expression in vivo does not depend critically on the ie1 function.

The lung tissues of the mice for which the T-cell frequencies were determined and those of another group of mice infected under the same conditions were explanted, and the incidence of reactivation in the cultures was investigated (Fig. 2C). At 4 and 7 months p.i., reactivation occurred in the lung tissues of all of the mice and of four of five mice infected with either the wt or the Δie1 virus, respectively, and at 12 months p.i., reactivation occurred in the lung tissues of six of seven mice infected with the Δie1 mutant and three of seven mice infected with wt MCMV. The reactivation incidences were also calculated based on the numbers of reactivation events observed in individual wells containing the lung pieces. When individual cultures of lung pieces explanted at 4 months p.i. were analyzed, the reactivation incidence was found to be high (70%) and almost identical for the wt virus and the Δie1 mutant (data not shown). At later time points, the reactivation incidence was much lower (10 to 40%), suggesting that fewer of the latent virus genomes were capable of reactivating then. At 4 and 12 months p.i., reactivation occurred in more of the cultures containing lung pieces from Δie1 mutant-infected mice than of the cultures with lung pieces from wt MCMV-infected mice. This was most likely due to a slightly higher genome load in the lungs of mice infected with the Δie1 mutant (compare, e.g., Fig. 5A). Altogether, these results confirmed that the ie1 gene is not needed for reactivation.

Induced reactivation of the Δie1 mutant in vivo. The latent infection is controlled by cellular immunity, and accordingly, recurrence of the MCMV infection is observed after ablation of immune cells by irradiation (46). To test whether the Δie1 mutant has the capacity to reactivate in vivo, infected mice were treated with hematoablative irradiation 4 months after infection. As an indicator of reactivation, we measured the increase in the viral genome copy number in the lungs 8 days after irradiation and compared these data to those for nonirradiated mice (Fig. 5A). Prior to irradiation, the copy number of latent viral genomes in infected lungs was slightly higher for the Δie1 mutant than for wt MCMV in this experiment. Following irradiation, the medians of the copy number of viral genomes increased similarly for both viruses (11-fold for wt MCMV and 8-fold for the Δie1 mutant). We also analyzed whether infectious virus was detectable in irradiated and nonirradiated mice. For the untreated mice, no virus was found in the lungs (Fig. 5B), as expected for a truly latent infection. However, after irradiation, infectious virus was present in the lungs of two of five mice infected with the Δie1 mutant and of four of five mice infected with wt MCMV. The identities of the recovered viruses were verified by PCR analysis (Fig. 5C). These experiments indicated that following hematoablative treatment of latently infected mice, MCMV is able to reactivate in vivo in the absence of the IE1 protein.

DISCUSSION

A detailed understanding of the molecular mechanisms of CMV reactivation is crucial for the development of specific therapeutic intervention strategies. In this study, we focused on the role of the ie1 gene in regulating the latency and reac-
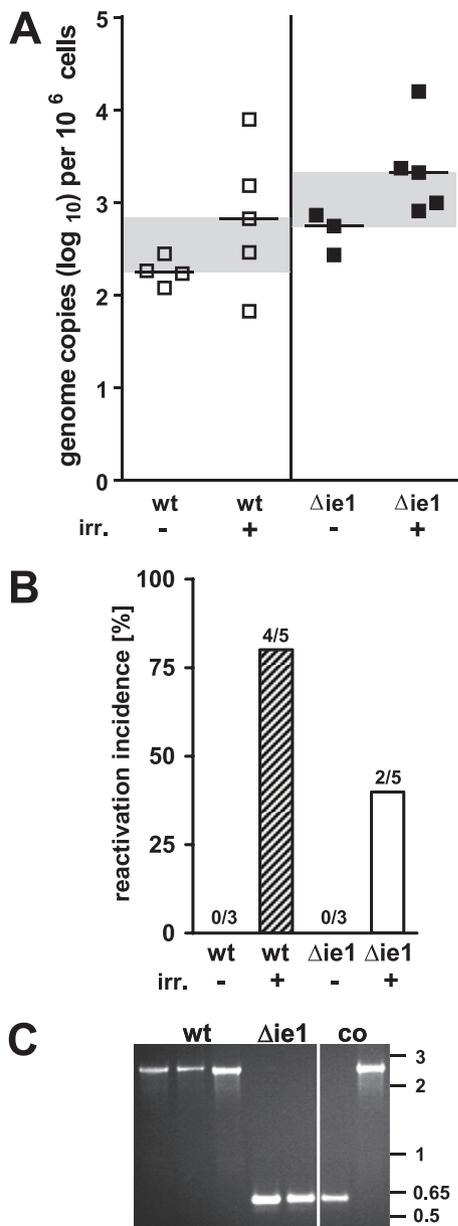
FIG. 5. In vivo reactivation of the Δie1 mutant in the lungs of irradiated mice. (A) Four months after infection with 10^2 PFU of wt MCMV or 10^4 PFU of the Δie1 mutant, mice were either irradiated (irr.) (+) or left untreated (−). Reactivation in the lungs was measured by an increase in the viral genome load (shaded areas). The genome loads of wt MCMV (□) and of the Δie1 mutant (■) per 10^6 lung cells are shown. Median values are indicated by horizontal bars. (B) The presence of infectious virus in the lungs of untreated or irradiated mice was analyzed by homogenizing the lung tissue and plating on fibroblast cultures. (C) PCR analysis was performed with DNA isolated from fibroblasts infected with the viruses recovered from irradiated mice (as shown in panel B) or from fibroblasts infected with the Δie1 mutant and wt MCMV as controls (co). The sizes of molecular mass markers (in kilobase pairs) are given on the right.

tivation of MCMV in vivo. Although the Δie1 mutant turned out to be attenuated in acute infection of newborn mice, it was able to establish a latent infection, and up to 12 months p.i. its reactivation capacity, as determined in lung explant cultures, was similar to that of wt MCMV. The load of viral genomes in

the lungs remained unchanged between 3 weeks and 7 months p.i., as was the case for wt MCMV, suggesting that the ie1 gene is also not required for the maintenance of the viral genomes during latency. Similarly, the Δie1 mutant reactivated in vivo after hematoablative irradiation, and the increase in number of a subset of MCMV-specific memory T cells, which may provide an indirect measurement of reactivation events in vivo, was in the same range for Δie1 mutant- and wt MCMV-infected mice. We conclude from these investigations that the ie1 gene is not essential for maintaining latency or for regulating the reactivation of MCMV.

Acute infection, establishment of latency, maintenance of the latent viral genomes, and reactivation are interconnected and interdependent phases of CMV infection. Accordingly, the extent of the primary CMV infection determines the number of viral genomes present in latency, and this in turn influences the risk of reactivation (43). Thus, to analyze the reactivation capacity of the Δie1 mutant, it was necessary to consider the preceding phases of infection. As expected from our previous study (10), the Δie1 mutant was found to be attenuated in comparison to the wt MCMV during acute infection of newborn mice. The cause of this attenuation was not the focus of this investigation, but our data and the observations of another group, who found that the doubling time for the titers of the Δie1 mutant was significantly delayed in several organs of irradiated mice, including the lungs (62), are consistent with our previous suggestion (10) that the Δie1 mutant displays increased sensitivity to the innate defense mechanisms of the host.

Infection with a nonlethal dose (100 PFU) of wt MCMV and a 10-fold higher dose of the Δie1 mutant led to comparable viral loads in the lungs during latency. This result could not be anticipated, since the acute infections did not proceed synchronously for the two viruses. While the titers of the Δie1 mutant at day 3 p.i. were higher than those of wt MCMV, the infection with the mutant was more rapidly controlled thereafter. It can be assumed that the virus has to attain high titers during acute infection in order to establish latency, possibly in rare cellular niches that may not be permissive for lytic replication. The decline in the number of viral genomes observed between acute and latent infection was more pronounced for the Δie1 mutant than for the wt virus; this could be due to a reduced ability of the Δie1 mutant to establish latency or, alternatively, to a saturation of the available cells supporting latency. The conclusion that we wish to draw, however, is that the Δie1 mutant is still capable of establishing latency.

The MCMV ie1 gene did not seem to be required for the maintenance of latent viral genomes, since the genome copy number did not decline during latency. This conclusion is also supported by the observation that the Δie1 mutant reactivated with an incidence similar to that of the wt virus at different time points during latency. At this time, we know very little about the mechanisms that govern the maintenance of the latent genomes. One possibility is the frequent recurrence of productive CMV infection, which would guarantee the replenishment of the pool of latently infected cells. Alternatively, the CMV genomes may be amplified and segregated during cell division of the latently infected cells, a mechanism employed mainly by gammaherpesviruses. In both cases, a change in the epigenetic modification of the chromatin structure of the viral genomes is

conceivable, and a role for the IE1 protein could have been expected.

The presence of viral genomes does not necessarily imply that a latent infection is established; it could also reflect an abortive infection that will never be able to recur. Thus, we analyzed the ability of the Δie1 mutant to reactivate by applying three different techniques to address this question: analysis of virus recovery from ex vivo lung explant cultures, monitoring of the memory inflation of MCMV-specific T cells, and induction of reactivation in vivo. In the ex vivo reactivation experiments, the Δie1 mutant displayed a high incidence of reactivation events at 4, 7, and 12 months p.i. It is clear that this assay is associated with some inherent variability; for instance, we observed fewer reactivation events for wt MCMV at 12 months p.i. than at the other time points, even when individual lung pieces were analyzed. We also have to consider that infection with the Δie1 mutant was performed with a 10- to 100-fold-higher dose to compensate for the reduced replication capacity of the mutant during acute infection. Infection with an identical inoculation dose would lead to a lower viral load of the Δie1 mutant and consequently would result in a reduced reactivation incidence. Overall, however, the data suggest that the Δie1 mutant is in principle able to establish latency, and when similar loads of latent viral genomes are present in the lung tissues, the mutant does not seem to be impaired in reactivation in comparison to wt MCMV.

The factors that induce reactivation in the explant cultures may include mechanical stress or hypoxia and may not necessarily reflect the physiological stimuli that trigger reactivation in vivo. Therefore, we also analyzed parameters that indicate in vivo reactivation. The maintenance or even expansion of CMV-specific memory T cells during the latent infection phase could be explained by frequent reactivation of viral protein expression in vivo, leading to the generation of antigens that trigger the proliferation of T cells (16, 17, 24, 49, 51). We therefore reasoned that analysis of the frequency of these T cells may give us at least a hint as to whether the capacity of the Δie1 mutant for reactivation in vivo is altered. Since one of the immunodominant epitopes of MCMV in BALB/c mice derived from the IE1 protein is missing in the Δie1 mutant, we focused on the T cells that are specific for the other immunodominant epitope, which originates from the m164 protein (17). Although there was quite a variation in the number of m164-specific T cells among individual mice at 12 months p.i., we did not see a significant difference in the frequencies of these T cells between Δie1 mutant- and wt MCMV-infected mice. We must point out that other mechanisms for the expansion of the CMV-specific memory T cells have been proposed (54) and that the exact mechanism for this phenomenon remains to be elucidated. However, it is reasonable to assume that frequent reactivation is the underlying principle that drives memory inflation (45, 51, 60). If this is true, then our data suggest that the Δie1 mutant is not impaired in transcriptional reactivation in vivo.

Finally, we analyzed the induced in vivo reactivation of the Δie1 mutant following ablation of the immune cells by irradiation. This resembles the situation in immunocompromised patients, e.g., in stem cell transplant recipients. After irradiation, increased levels of viral genomes were found, and infectious virus was recovered from the lungs of mice infected with either virus, clearly demonstrating that the Δie1 mutant can reactivate in vivo. The increases in the viral genome loads after irradiation were similar for the two viruses. Infectious virus was, however, recovered in fewer mice infected with the Δie1 mutant than with the wt virus. This may be interpreted as a reduced reactivation capacity of the Δie1 mutant, but it is rather attributable to the limited sensitivity of the assay. It has been shown previously that virus reactivation after irradiation is a stochastic event and does not necessarily result in reactivated virus in each organ (43). In addition, neutralizing antibodies confine the reactivated virus to small foci where the reactivation has occurred (23), and therefore it was not surprising that we could detect small amounts of virus in the lungs of some irradiated mice only. Further investigations to quantify the reactivation capacity of the Δie1 mutant in vivo may have to be performed with mice lacking antibodies (40).

The salient result of our study, namely, that the Δie1 mutant is able to reactivate in explanted lung tissue and also in vivo, was unexpected, since in a previous study the MCMV IE1 protein was clearly shown to interact with the histone deacetylase HDAC2 at an early stage of the lytic infection cycle in vitro, and this interaction correlated with a marked reduction in the deacetylation activity in infected cells (55). In addition, the IE1 protein interacts with the repressor proteins PML and Daxx and may inhibit their function, thus promoting the viral gene expression program. This begs the question of why the Δie1 mutant showed no phenotype during reactivation, at a stage of the CMV infection when the antiviral defense of the latently infected cell must be overcome. One explanation is that other viral proteins substitute for the regulatory function of the IE1 protein. Experiments by Tang and Maul indicated that IE1 is indeed not the sole MCMV protein that inhibits HDAC activity (55). In HCMV, both IE1 and IE2 are able to interact with HDAC3 (37). Thus, it is reasonable to assume that the homolog of the HCMV IE2 protein in MCMV, the IE3 protein, might possess a similar property, but this remains to be examined. If IE3 inhibits histone deacetylases as well, this would perfectly explain the compensation of the IE1 function.

An important question is whether the result that we obtained for the MCMV ie1 gene can be extrapolated to the role of the HCMV ie1 gene in the reactivation of HCMV. IE1 proteins of MCMV and HCMV share many functions, including the ability to bind and to inhibit histone deacetylases and to disperse proteins that are associated with PML NBs (reviewed in references 7 and 31). Both IE1 proteins are multifunctional, and the dissection of their various functions and their assignment to different domains of the proteins have just begun (18, 28, 31, 48, 56, 62). Concerns have been raised about differences in the functions of the major IE proteins of MCMV and HCMV and hence about the validity of the MCMV model (31). However, the recent finding that the MCMV IE3 protein is able to arrest the cell cycle in infected cells, as is its HCMV homolog, IE2 (61), underlines the fact that the CMV major IE proteins have much more in common than previously anticipated. Another recently described property of the HCMV IE1 protein is its interaction with the signal transducer and activator of transcription STAT2, interfering with interferon signaling and the activation of interferon-stimulated genes (18, 39). It is not yet known whether the MCMV IE1 protein possesses a similar role in combating the cellular innate immune re-

sponse, but this would explain the observed attenuation of the Δie1 mutant in vivo (10, 62; also this study).

In summary, we were able to show that the function of the MCMV ie1 gene is not critical for the maintenance of latency or for reactivation. In order to learn more about the role of the IE1 protein in the pathogenesis of CMV, we have to dissect the different functions of the MCMV and HCMV IE1 proteins more precisely. By constructing MCMV recombinants with more subtle mutations in the ie1 gene, we can test further hypotheses about the function of the IE1 protein in vivo.

ACKNOWLEDGMENTS

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