

Latency, without Persistence, of Murine Cytomegalovirus in the Spleen and Kidney

JESSICA L. POLLOCK AND HERBERT W. VIRGIN IV*

Center for Immunology and Division of Infectious Diseases, Departments of Medicine, Pathology, and Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110

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It is not known if murine cytomegalovirus (MCMV) establishes a state of molecular latency independent of low-level persistent infection. The presence of low levels of infectious MCMV distinguishes persistence from molecular latency. Thus, the distinction between persistence and latency has depended on the sensitivity of plaque assays for detecting low levels of infectious virus in tissue of previously infected mice. To determine whether MCMV establishes molecular latency or remains persistent, we developed two assays for detecting low levels of MCMV in tissue. Using prolonged *in vitro* culture of virus with either mouse embryonic fibroblasts or the murine 3T12 fibroblast cell line, we reproducibly detected a single PFU of MCMV. Inclusion of undiluted sonicated tissue in this assay decreased sensitivity by up to 100-fold. However, sensitivity was improved to 1 PFU of MCMV when sonicated tissue was appropriately diluted. Severe combined immunodeficient (SCID) mice were also used to detect MCMV in sonicated tissue. Infection of SCID mice with a single PFU of MCMV killed two of eight SCID mice, and the 50% lethal dose of MCMV in SCID mice was 2 to 3 PFU. Applying these two methods, we detected infectious virus in 0 of 34 spleens, 1 of 34 kidneys, and 0 of 37 salivary glands from latently infected mice. Spleens and kidneys assessed for persistent virus contained MCMV DNA by PCR and reactivated after 10 to 50 days in explant cultures. Latently infected kidney cells reactivated after adoptive transfer to SCID mice. Quantitation of the MCMV genome by PCR showed that latently infected spleens without detectable infectious MCMV contained about 3,000,000 copies of the MCMV genome. These results demonstrate that MCMV latency in spleen and kidney exists in the absence of low-level persistent infection. Use of assays with defined sensitivity for detection of MCMV in tissue provides a basis for evaluation of cytomegalovirus gene expression in the spleen and kidney during molecular latency.

Human cytomegalovirus (HCMV) is a significant pathogen during immunosuppression and pregnancy (4, 21, 23). HCMV recrudescence in these settings may be due to reactivation of virus from latency. However, occasional prolonged shedding of HCMV and the difficulty in quantitative detection of infectious HCMV raise the possibility that HCMV is a persistent virus. Murine cytomegalovirus (MCMV) infection has been used extensively as a model for analysis of cytomegalovirus latency. MCMV is a suitable animal model for HCMV (reviewed in reference 27) in that these viruses have (i) similar genomes and genetic organization, (ii) analogous gene products with similar functions, (iii) comparable, although not identical, pathogenesis and tropism, and (iv) biologically similar latency and reactivation (see below).

Acute infection with MCMV is rapidly cleared (as measured by plaque assay of tissue homogenates) from visceral organs. However, persistent infection continues in salivary gland acinar cells for a period of up to several months (7, 25). When virus is cleared from salivary gland, MCMV DNA is detected by PCR in many organs, including the spleen, lung, kidney, and salivary gland (2, 14, 19). MCMV-induced disease occurs when mice which have recovered from acute infection are immunosuppressed (2, 9, 12, 24). In addition, infectious MCMV ap-

pears after latently infected spleens are explanted *in vitro* (11, 18).

The distinction between molecular latency (absence of infectious virus) and persistence (low-level production of infectious virus) has primarily relied on the sensitivity of plaque assays for detecting infectious MCMV in tissue. The importance of plaque assay sensitivity for distinguishing between persistent infection and latency has been emphasized (10, 18). Some studies have used plaque assays with incompletely defined sensitivity to examine latently infected tissues for low levels of infectious virus (17, 24, 28). One recent study using mouse embryonic fibroblast (MEF) monolayers defined sensitivities of detection of 15 to 20 PFU in salivary gland (18). Others used long-term cocultures of entire sonicated organs with permissive cells to demonstrate a lack of infectious virus in latent tissue (2, 8). This is the strongest evidence to date that supports molecular latency of MCMV. However, two recent publications demonstrate the presence of transcripts derived from the major immediate-early (MIE) locus of MCMV (ie1 transcripts) in mice following recovery from acute MCMV infection (6, 29). Since products of the MIE region are likely critical for viral replication, the presence of MIE transcripts was used to argue for MCMV persistence below the level of plaque assay sensitivity (29). Thus, there is disagreement as to whether MCMV is truly latent, persistent, or both after recovery from acute infection.

In this report, we define the sensitivity of two assays for detecting low levels of MCMV in tissue. We combined these two detection assays with quantitation of the MCMV genome and reactivation of latent virus *in vitro* and *in vivo* to demon-

* Corresponding author. Mailing address: Center for Immunology and Division of Infectious Diseases, Departments of Medicine, Pathology, and Molecular Microbiology, Washington University School of Medicine, Box 8051, 660 S. Euclid Ave., St. Louis, Mo. 63110. Phone: (314) 362-9223. Fax: (314) 362-9333. Electronic mail address: virgin@visar.wustl.edu.

strate that MCMV latency does not require continuous production of infectious virus.

MATERIALS AND METHODS

Cells and tissue culture media. All media contained 100 U of penicillin and 100 µg of streptomycin (Biofluids, Rockville, Md.) per ml, 1% glutamine (Biofluids), and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Biofluids). Tissue culture-passaged MCMV stocks were grown in Dulbecco's modification of Eagle's medium (DMEM; Mediatech, Washington, D.C.)–5% fetal calf serum (FCS; HyClone, Logan, Utah) and stored in DMEM–10% FCS with 10% dimethyl sulfoxide (Fisher, Fair Lawn, N.J.). 3T12 murine fibroblasts (ATCC 164-CCL) were passed in DMEM–2.5 to 5% FCS. MEFs were generated by cultivation of minced CB17 mouse embryos (days 14 to 16) in DMEM–10% FCS. MEFs were used during initial passaging or thawed from frozen stocks. All MEFs were used before the fifth passage.

Viruses and virus stock preparations. MCMV Smith strain was obtained from the American Type Culture Collection (VR-194, lot 10). MCMV stocks were generated from infected 3T12 cell supernatants as previously described (5). Salivary gland-passaged MCMV stocks were made from salivary glands of 8-week-old BALB mice infected intraperitoneally (i.p.) with 10⁵ PFU of MCMV. Salivary glands were harvested on day 17 of infection, Dounce homogenized on ice, and adjusted to a 1/10 (wt/vol) suspension with DMEM–10% FCS–10% dimethyl sulfoxide. Virus stocks were stored at –80°C.

Mice, mouse infections, and organ harvests. Immunocompetent mice were purchased (BALB/cANNCR; National Cancer Institute, Frederick, Md.) or bred at Washington University (CB17). To establish latency, mice were infected i.p. with 10⁷ PFU of MCMV or 10⁴ PFU of salivary gland-passaged MCMV and rested for 2 to 8 months. Serology results showed that some mice had been exposed to murine hepatitis virus prior to experimentation. All parts of molecular latency experiments were repeated in seronegative animals. Results from seronegative and seropositive mice were identical, and results were pooled. CB17 and CB17 severe combined immunodeficient (SCID) mice (3) were bred at Washington University by serial brother-sister mating. For MCMV 50% lethal dose experiments and molecular latency studies, SCID mice were injected i.p. with medium, MCMV diluted in DMEM–10% FCS, or 1 ml of sonicated tissue, using a 22-gauge needle. In 50% lethal dose experiments, mortality always occurred on or before day 28. Salivary glands of survivors were titered 33 days after infection to confirm the absence of MCMV. For molecular latency studies (Table 1), salivary glands from SCID mice were harvested 14 days after i.p. injection of sonicated tissue and cultured to detect MCMV infection. Organs were harvested into 1 ml of DMEM–10% FCS–10% dimethyl sulfoxide. For molecular latency studies of spleen and kidney, 10% of each organ was frozen for PCR analysis (see below), 20% of each organ was explanted to assess reactivation (see below), and the remaining 70% of each organ was pooled. Data for the spleen and kidney are presented per latently infected organ on the basis of correction for the amount of each organ used for PCR and explantation. Thus, 10 spleens were processed to yield 7 spleen equivalents for molecular latency studies. When salivary glands were assayed for persistent virus, entire lobes were pooled and tested in either of the two detection assays. Prior to sonication, pooled minced organs were always divided into groups so that 5 to 25 PFU of MCMV could be added to some to serve as positive controls.

MCMV plaque assay. 3T12 cells (2 × 10⁵ per well) were plated and incubated overnight in six-well clusters (Costar, Cambridge, Mass.). Cells were approximately 90% confluent at the start of the plaque assay. Tissue samples were minced, sonicated (Branson Sonifier model 250; Branson Ultrasonics Corporation, Danbury, Conn.) at 30 to 35 W for 15 to 20 s, and stored on ice prior to infection (sonic extract). Infection and overlays were performed as previously described (5).

In vitro culture assay for detection of MCMV. Prior to infection, 10⁴ 3T12 cells or MEFs were plated per well in 96-well plates (Costar). When monolayers were 90% confluent, medium was aspirated and wells were inoculated with 20 µl containing 0.01 to 10 PFU of MCMV. After 1 h at 37°C and 5% CO₂, 200 µl of DMEM–10% FCS per well was added without removing the inoculum. Monolayers were scored daily for 14 days for viral cytopathic effect (CPE) with medium changes every 5 days. To confirm absence of MCMV after 14 days, cultured media from wells without CPE were transferred to fresh 3T12 monolayers, which were scored for CPE after 6 days. Since the stability of MCMV to sonication was demonstrated at all intensities used in these experiments both in the presence and in the absence of tissue (not shown), all data generated in experiments which compared sonication with no sonication are pooled for presentation.

For detection of low levels of persistent MCMV in whole organs, the assay was scaled up by plating 10⁶ MEFs in 75-cm² flasks (Sarstedt, Newton, N.C.). Organs were sonicated in 1 ml of DMEM–10% FCS and then raised to 10 ml (1:10 dilution). To keep sonicated tissue at a noninhibitory concentration, each of three T75 flasks was inoculated with 3.3 ml of sonicated organ diluted 1:10. After a 1-h incubation, 30 ml of DMEM–10% FCS was added to each flask (final organ dilution, 1:100). To minimize toxicity, salivary gland samples were centrifuged (13,800 × g) at 4°C for 2 h, and the pellet was resuspended in the original volume with fresh DMEM–10% FCS prior to 1:10 dilution and cocultivation on MEFs. Flasks were monitored for 14 days for viral CPE. The absence of MCMV in a

flask without CPE was confirmed by inoculation of the medium onto fresh 3T12 monolayers.

Detection of MCMV DNA by nested PCR. Organ DNA from naive or latent animals was prepared with a QIAamp tissue kit (Qiagen, Chatsworth, Calif.) or by standard overnight digestion at 56°C in the presence of 1% sodium dodecyl sulfate (SDS) and proteinase K (0.7 mg/ml), phenol-chloroform extraction, and ethanol precipitation. Amplification results were identical for the two methods (not shown). The MCMV genome was quantitated by dilutional nested PCR. DNA samples were adjusted to 0.5 µg/µl in TE (10 mM Tris, 1 mM EDTA [pH 8]), and serial 10-fold dilutions were performed with carrier DNA (prepared from naive mouse liver) or tRNA (Sigma, St. Louis, Mo.) at 0.5 µg/µl in TE. Each PCR mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM MgCl₂, 0.2 mM nucleotides, 0.15 µM each primer, and 1 U of *Taq* (*Thermus aquaticus*) DNA polymerase. Reactions were performed on a Temp Tronic thermocycler (Barnstead/ThermoLynce, Dubuque, Iowa). Primers were derived by using the *ie1* sequence (13) and OLIGO primer analysis software program (National Biosciences Inc., Plymouth, Minn.). Primer locations correspond to numbering within the published *ie1* sequence (13). PCR primers were chosen so that cleavage at the *Hind*III site in the *ie1* gene should generate 394- and 170-bp fragments from the 564-bp product of nested PCR. Outer primers were 5'-CTAGCCAATGATATCTTCGAGCG3' (1742 to 1764) and 5'-GTGCTAGATTGTATCTGGTCTCCTC3' (2357 to 2332, antisense). Inner primers were 5'-AAAGACAACGCAAGATGATATACG3' (1770 to 1793) and 5'-TCA-GATCAGCTAAGGTAGCCAAG3' (2333 to 2311, antisense). The initial round of PCR was performed with 25 cycles of 95°C for 1 min, 54.9°C for 1 min, and 70°C for 1 min. The second round of PCR had identical conditions except that the annealing temperature was 56°C and the reaction was amplified for 35 cycles. Both programs began with an initial denaturation at 95°C for 5 min and ended with a 1-min final extension at 70°C. Initial PCR mixtures always contained 1 µg of total nucleic acid (sample plus carrier). Four percent of the initial PCR product was used as a template for second-round PCR with the inner primers. Every PCR assay included a positive control, a carrier nucleic acid-only control, and a no-DNA/RNA control in which 2 µl of water was added to the reaction tube. PCR products were separated on a 2% agarose gel and visualized by Southern blotting (see below) or ethidium bromide staining. Tissue samples which were negative for MCMV DNA were always positive when entered into a PCR with primers for β-actin to ensure integrity of DNA. PCR conditions for actin were identical to those described above, with the following exceptions. One round of PCR was performed with 40 cycles of 95°C for 1 min, 63°C for 1 min, and 70°C for 1 min. Murine β-actin primer locations correspond to the published sequence (26). Primer sequences 5'-CTGGTCTGACACAGGCATTGTGA TG3' (514 to 539) or 5'-TGCTCTCCCTACGCCATCCTGTG3' (587 to 609) and either 5'-CCAACCAACTGCTGTGCGCTTAC3' (1436 to 1413, antisense) or 5'-TTACACAGAAGCAATGCTGTACCTTCC3' (1668 to 1641, antisense) were selected by using the OLIGO primer analysis software program.

Quantitation of MCMV genome in latently infected tissue by PCR. Plasmid pAMB25 containing the *ie1* gene (provided by U. Koszinowski) was used to determine the sensitivity of our nested PCR for detection of *ie1* DNA (15). pAMB25 was isolated (Wizard Minipreps DNA purification system; Promega, Madison, Wis.), purified by multiple phenol-chloroform extractions, quantitated spectrophotometrically, and diluted in mouse liver DNA or tRNA (0.5 µg/µl) in TE. One microgram of total DNA (pAMB25 plus carrier) from serial 10-fold dilutions of pAMB25 in mouse liver DNA or tRNA was entered into nested PCR to detect *ie1* sequences (see above). For Southern blots, 20 µl of a 50-µl PCR mixture was run on a 2% agarose gel and transferred to Magna Graph nylon transfer membranes (Micron Separations Inc., Westboro, Mass.). Southern blotting was performed by standard methods (22). To generate an *ie1*-specific probe, 0.3 µg of pAMB25 was entered into a PCR and amplified with the outer set of primers. The amplified *ie1* sequence was isolated by gel purification, labelled with ³²P by random priming (Megaprime DNA labelling system; Amersham, Arlington Heights, Ill.), and used as the probe. Membranes were washed in 1 × SSC (0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS for 4 to 6 h at 65°C prior to autoradiography. To determine the number of MCMV genomes in organs from latently infected animals, we performed nested PCR on serial 10-fold dilutions of tissue DNA (from 10 pg to 1 µg) in carrier mouse liver DNA or tRNA (see above). When converting the amount of latent cell DNA from nanograms per reaction to cell equivalents per reaction, we assumed 2.7 × 10⁹ bp per haploid mouse genome (16). The range of latent DNA tested per reaction corresponded to ~1 to 172,000 cell equivalents of DNA per reaction. Reaction products were electrophoresed and stained with ethidium bromide to detect *ie1*-specific product.

Reactivation in vitro. Fragments of spleens and kidneys used in molecular latency studies were explanted onto MEF monolayers and assessed every other day for CPE in the indicator monolayer. One to two tissue fragments (1 to 3 mm³ in size, about 20% of the whole organ) were cultured per well per latent organ. Explants were maintained in DMEM–10% FCS with medium changes every 2 to 3 days for at least 50 days. Reactivation was confirmed by transfer of medium from explant wells with CPE to fresh 3T12 monolayers, which were observed for CPE. Medium from all wells without CPE after 50 days was transferred to 3T12 monolayers to confirm the absence of infectious MCMV.

Reactivation in SCID mice. Preliminary data showed that MCMV reactivation was detected 24 to 33 days after adoptive transfer of single-cell suspensions from

latently infected kidney to SCID mice (not shown). Kidneys from 10 mice recovered from acute MCMV infection were used to assess whether organs evaluated for low-level persistent infection reactivated *in vivo*. For this experiment, one kidney from each mouse was evaluated for persistent infection and cells from the other kidney were transferred to SCID mice, which were subsequently sacrificed and evaluated for evidence of active MCMV infection. Kidneys to be tested for reactivation in SCID mice were minced with scissors and triturated with a 10-ml syringe and 18-gauge needle to generate a single-cell suspension. One milliliter of this suspension containing 2×10^7 cells in DMEM-10% FCS was injected *i.p.*, and mice were sacrificed 28 days later. Salivary glands were removed, minced, sonicated, centrifuged as described above, resuspended in medium, and inoculated at 1:10 dilution on three 75-cm² flasks with a 3T12 monolayer. MCMV CPE was evident after 2 to 3 days.

RESULTS

Sonicated tissues inhibit detection of MCMV by plaque assay. We compared plaque assay sensitivities for detection of MCMV in sonicated tissues and medium-alone controls. Undiluted spleen or kidney sonic extracts inhibited detection of MCMV by >90% (not shown). When sonicated tissues were diluted 1:10 with medium prior to inoculation, the inhibition by spleen and kidney tissue, but not salivary gland, disappeared. Sonicated tissue did not destroy infectious MCMV, since we were able to recover infectious virus from inhibitory sonicated tissue by centrifugation and resuspension of the pellet in fresh medium prior to plaque assay. In addition, when 10^4 or 10^8 PFU was added to undiluted sonicated tissue and serial 10-fold dilutions of samples were subjected to plaque assay, we were able to quantitatively detect input virus (not shown). These results show that lack of detection was due to inhibition rather than destruction of infectious virus in the presence of sonicated tissue. Therefore, the presence of low levels of MCMV in sonicated tissue could be missed by reliance on detection by plaque assay unless tissue samples were adequately diluted. As dilution of organ sonic extracts 1:10 prior to inoculation and to a final dilution of 1:100 would require inoculation of 50 to 100 wells per organ evaluated, we sought a more practical assay with defined sensitivity for evaluation of whole organs for low-level persistent MCMV.

In vitro culture assay sensitivity and detection of MCMV in sonicated tissue. MEFs or 3T12 cells were infected with various doses of MCMV in 96-well plates and cultured for 14 days. Virus infection was scored by characteristic MCMV CPE. The accuracy of viral detection using CPE in 96-well plates was tested in early experiments by culturing supernatant from wells with signs of CPE and showing that these wells contained infectious virus. To quantitate the sensitivity of this assay, dilutions of virus were prepared so that inocula contained between 0.01 and 10 PFU of MCMV (Fig. 1). Inocula containing an average of 1 PFU per well infected 90% of wells with MEF monolayers, compared with 75% of wells with 3T12 monolayers (Fig. 1). The percentage of positive wells on 3T12 monolayers is concordant with the expectation from a Poisson distribution, which predicts that inoculation with 1 PFU per well should result in infection in 63% of wells with 1 or more PFU, with 37% of wells remaining uninfected (1). MEFs were more sensitive for detection of MCMV than 3T12 cells when 1 PFU was detected. However, when less than 1 PFU was added per inoculum, MEFs and 3T12 cells were comparably sensitive. Thus, in the absence of tissue extracts, this assay can quantitatively detect MCMV.

We next evaluated detection of MCMV in the presence of sonicated tissue (Fig. 2). As in the plaque assay, detection of MCMV was significantly less sensitive in the presence of undiluted spleen or kidney (Fig. 2A and C). Dilution of sonicated tissue (1:10 prior to inoculation and final dilution of 1:100) removed the inhibitory activity of spleen sonic extracts and

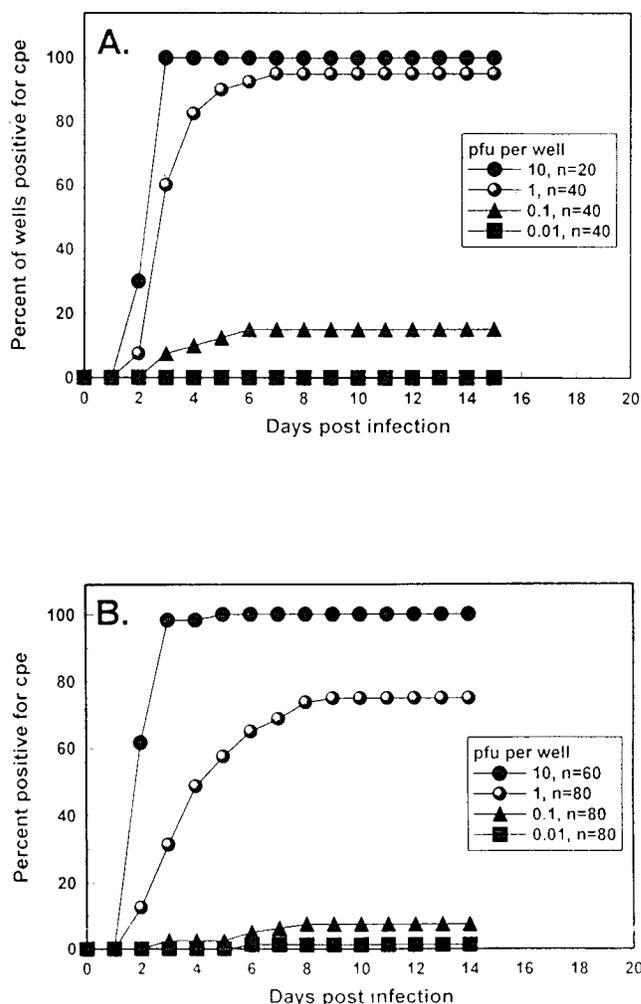


FIG. 1. Detection of MCMV in long-term culture assay. Dilutions of MCMV containing 0.01 to 10 PFU in 20 μ l of DMEM were made. Ten wells of either MEFs or 3T12 cells were infected with each dilution, and every dilution was tested in two to eight experiments. (A) MEF monolayers; (B) 3T12 monolayers. The number of wells scored for each experimental condition is shown in the inset.

significantly improved detection of MCMV in kidney sonic extracts (Fig. 2B and D). When salivary gland samples were diluted 1:10 and centrifuged at $13,800 \times g$ and the pellet was resuspended in the original volume with fresh medium, 1 PFU of MCMV was detected in 24 of 40 wells, or 60% of the time. Thus, appropriate dilution of sonicated tissue, with or without centrifugation, allowed us to detect MCMV quantitatively in organ sonic extracts. To detect low levels of infectious MCMV in entire latent organs, we scaled up the assay defined above by inoculating 90%-confluent MEFs in three 75-cm² flasks with one organ equivalent (final dilution, 1:100). As expected, a single PFU of MCMV was detected in this scaled-up assay when inocula were diluted 1:10 (not shown).

SCID mice for detection of low levels of MCMV in tissue. Both the plaque assay and long-term culture assays (see above) depend on *in vitro* culture of cells to detect infectious virus. We wanted an assay which did not rely on *in vitro* culture to detect MCMV in organs. We evaluated SCID mice, lacking functional T and B cells (3), for detection of low levels of infectious MCMV in tissue samples. Forty-six SCID mice were injected

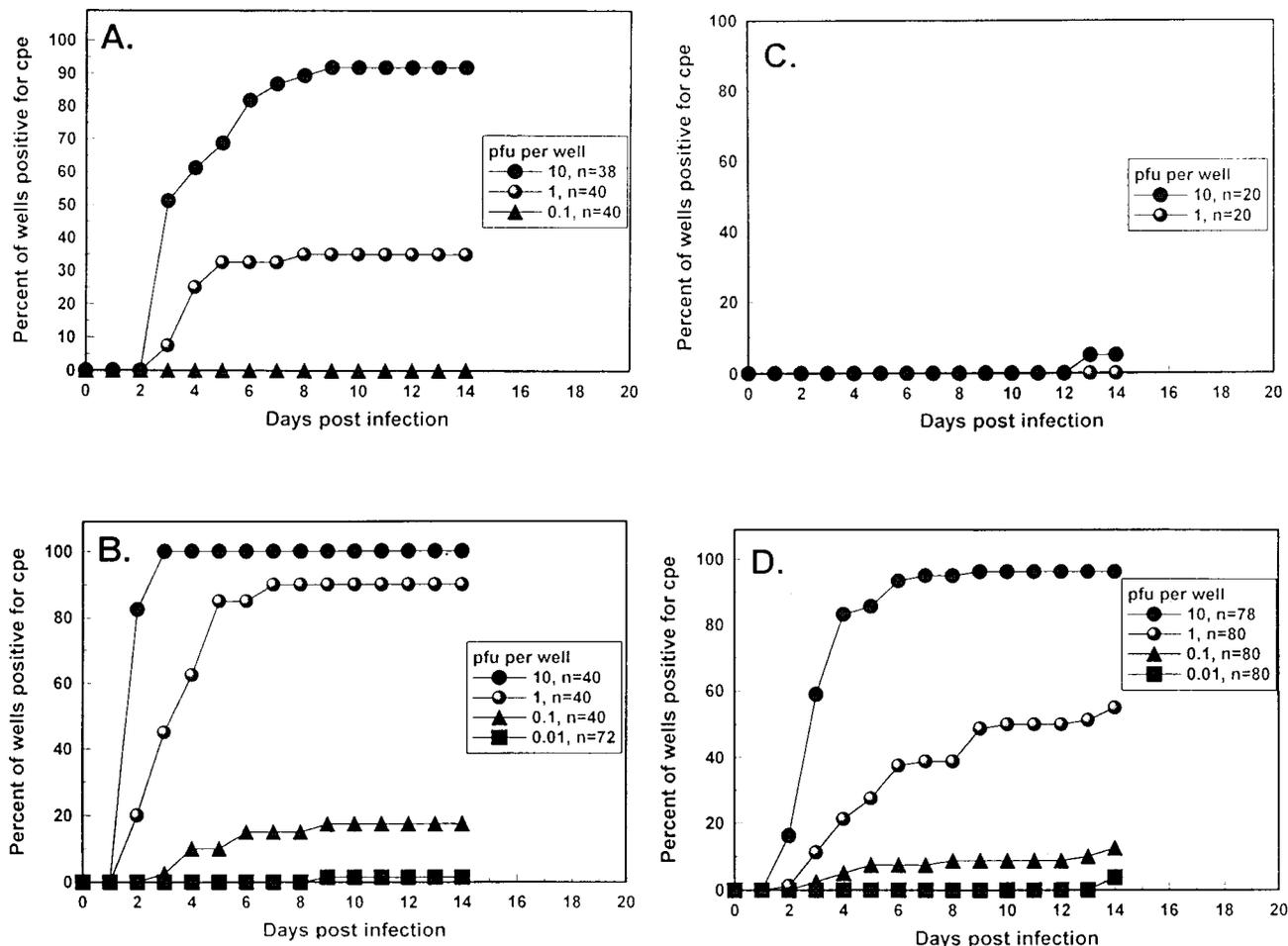


FIG. 2. Detection of MCMV in the presence of sonicated tissue. Dilutions of MCMV containing 0.01 to 10 PFU in either undiluted or diluted sonicated spleen or kidney were made. In each experiment, 0.01 to 10 PFU/20 μ l was added to a 1-ml aliquot of each tissue with or without subsequent sonication and used to infect 3T12 monolayers in 96-well plates. Each dilution of MCMV in each tissue sonic extract was plated onto 10 wells, and each line represents two to eight individual experiments. Medium-alone controls were performed with each experiment and gave results comparable to those in Fig. 1. (A) Detection of MCMV in undiluted spleen sonic extract; (B) detection of MCMV in spleen sonic extract diluted 1:10 in DMEM-10% FCS prior to inoculation and 1:100 after infection; (C) detection of MCMV in undiluted kidney sonic extract; (D) detection of MCMV in kidney sonic extract diluted 1:10 in DMEM-10% FCS prior to inoculation and 1:100 after infection.

i.p. with 0.01 to 9,000 PFU of MCMV, as defined by MCMV titer from 3T12 plaque assay, and monitored mortality for 28 days. Virus titers in salivary glands of all survivors were determined to confirm the absence of MCMV. Four mice were used per dose of $\geq 1,000$ PFU, and eight to nine mice were used per dose of ≤ 100 PFU of MCMV. Infection with an average of 1 PFU per mouse killed two of eight SCID mice. Using Reed-Muench analysis (20), we found that the MCMV 50% lethal dose for SCID mice was 2 to 3 PFU. Thus, SCID mice are sensitive indicators for infectious MCMV.

Molecular MCMV latency in immunocompetent mice. Using both in vitro culture and SCID mice, we evaluated immunocompetent mice 2 to 8 months postinfection for low levels of infectious MCMV in sonicated spleen, kidney, and salivary gland. Transferring 1 to 1.5 sonicated organ equivalents i.p. into each of 48 SCID mice, we tested the equivalent of 16 spleens, 21 kidneys, and 19 salivary glands, and none resulted in MCMV infection (Table 1). MCMV infection occurred in two of six SCID mice 14 days after i.p. injection with undiluted sonicated tissue supplemented with 10 PFU of MCMV. When 10 to 25 PFU of MCMV was added to undiluted organs and sonicated prior to injection, 14 of 18 SCID mice had detectable

MCMV infection by day 14 (Table 1). By using MEF monolayers in vitro, infectious MCMV was detected in 0 of 18 latently infected spleens, 1 of 13 latently infected kidneys, and 0 of 18 latently infected salivary glands, while 5 PFU of MCMV

TABLE 1. Lack of dependence of latency on low-level persistence of MCMV in organs

Latently infected organ	Detection of MCMV in organs (no. positive/no. tested)		
	In vitro culture on MEFs	Adoptive transfer to SCID mice	Reactivation from explants
Spleen	0/18	0/16	37/37
Spleen + MCMV ^a	6/6	5/6	ND ^b
Kidney	1/13	0/21	27/41
Kidney + MCMV ^a	4/5	4/6	ND
Salivary gland	0/18	0/19	ND
Salivary gland + MCMV ^a	4/6	5/6	ND

^a MCMV was added at 5 PFU for culture on MEFs and 10 to 25 PFU for transfer to SCID mice.
^b ND, not done.

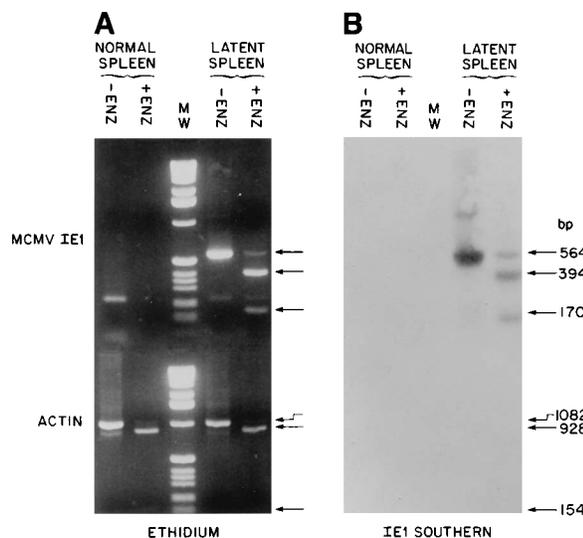


FIG. 3. Detection of MCMV ie1 DNA in latent tissues. (A) Ethidium bromide-stained agarose gel of PCR products from reactions specific for MCMV ie1 or actin DNA. DNA products were digested with restriction endonuclease *Hind*III, which is predicted to cut the amplified 564-bp fragment of ie1 into 394- and 170-bp fragments, or *Xba*I, which is predicted to cut the amplified 1,082-bp fragment of β -actin into 928- and 154-bp fragments. PCR of normal spleen DNA with actin primers resulted in a product which was visible by ethidium bromide staining. (B) Southern blot. The ie1 region of pAMB25 was amplified by PCR, labeled with 32 P, and used as a probe to further confirm the identity of the ie1 product in latent tissue and the absence of the product after PCR of normal spleen. ENZ, enzyme; MW, molecular weight markers.

added to identical organ equivalents was detected in 82% (14 of 17) of cultures (Table 1). Since prolonged culture with MEFs can detect 1 PFU in appropriately diluted sonicated tissue, these results show that the great majority of latently infected organs do not contain infectious MCMV. After correcting for the fraction of each spleen and kidney used in PCR analysis and reactivation studies, we assayed the equivalent of 34 spleens, 34 kidneys, and 37 salivary glands for the presence of low-level persistent infections by these two methods. Of the 35 organ equivalents to which 5 to 25 PFU of MCMV was intentionally added, 77% (28 of 35 organs) yielded MCMV infections by these two assays.

MCMV reactivates from latently infected spleens and kidneys. Explants were initiated from spleens and kidneys used in all molecular latency studies. Cultured fragments of both spleen and kidney explants yielded infectious virus 10 to 50 days after explantation (Table 1). One hundred percent (37 of 37) of splenic explants and 66% (27 of 41) of kidney explants reactivated by day 50. To test for reactivation *in vivo*, 2×10^7 unsonicated kidney cells from latently infected mice were transferred *i.p.* into SCID mice. The titers were determined for MCMV from salivary glands of 10 of 10 SCID recipients on day 28 after transfer. While the SCID mouse is a suitable environment for reactivation of MCMV from transferred kidney cells, we have not observed reactivation of MCMV after transfer of 2×10^6 to 1×10^8 spleen cells from latently infected mice (not shown).

Quantitation of MCMV DNA in spleens and kidneys. We used PCR to quantitate the level of MCMV genome present in the same spleens and kidneys evaluated for persistent infection and reactivation studies (see above). We confirmed the specificity of our ie1 PCR product by Southern blotting and digestion with *Hind*III (Fig. 3). Naive tissues were always negative for amplification of the ie1 gene (Fig. 3). While nested PCR

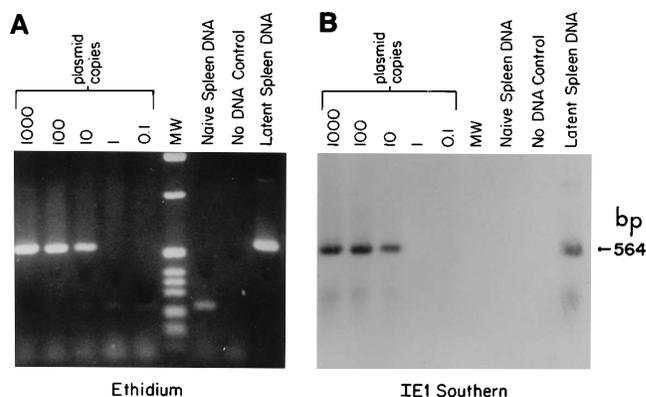


FIG. 4. Sensitivity of detection of ie1 DNA in nested PCR. (A) Ethidium bromide-stained agarose gel of reaction products following nested PCR. Notation above each lane indicates the number of input copies of pAMB25. A total of 1 μ g of carrier DNA with pAMB25, naive spleen DNA, or latently infected spleen DNA was added to each reaction mixture. Naive spleen DNA showed amplification of β -actin when tested in a PCR with actin primers (not shown). (B) Southern blot. PCR-amplified ie1 sequence from pAMB25 was labeled with 32 P and used as the probe. MW, molecular weight markers.

with naive mouse DNA resulted in a nonspecific product of approximately 240 bp, this fragment did not hybridize to the ie1 probe in the Southern blots (Fig. 3B and 4B) and disappeared when tRNA was used as the carrier nucleic acid (not shown). Tissue DNA samples which tested negative for MCMV DNA always tested positive in a PCR with β -actin primers (Fig. 3 and data not shown). Using this assay, we have detected the MCMV genome in the lungs, hearts, livers, spleens, kidneys, and brains, but not the blood, of latently infected mice (data not shown).

To determine the sensitivity of ie1 detection, known amounts of pAMB25 were entered into the nested PCR assay (Fig. 4). Ten copies of pAMB25 were detected 91% of the time (31 of 34 reactions), while one copy was detected 22% of the time (5 of 22 reactions). Southern blotting did not enhance the level of detection (Fig. 4B). The sensitivity of the assay was the same whether linearized or supercoiled plasmid was used and was not affected by the presence of carrier DNA (not shown). To ensure that MCMV DNA was not lost or damaged during DNA preparation from tissue, 10^4 copies of pAMB25 were added to naive spleen fragments prior to cell lysis and DNA extraction. Assuming total recovery of plasmid, samples were diluted to contain 10 copies of plasmid and tested by nested PCR. MCMV DNA was detected in 11 (73%) of 15 samples assayed. Since this level of detection is similar to that seen with plasmid diluted in carrier DNA (see above), our DNA isolation procedures quantitatively recover added plasmid containing MCMV sequences.

We assayed ~ 1 to 172,000 cell equivalents (0.01 to 1,000 ng) of DNA for the MCMV genome. We evaluated DNA samples from 20 latently infected spleens and 24 kidneys and summarized the percentage of positive reactions at each dilution of latently infected organ DNA (Fig. 5). Within each dilution series, the final dilution from which amplification of ie1 occurred was assumed to contain ≥ 10 copies of ie1 DNA. Fifty percent of spleen DNA samples gave a positive signal (≥ 10 copies of ie1) when 2 ng (~ 350 cell equivalents) of DNA was subjected to PCR. Fifty percent of kidney DNA samples amplified MCMV DNA (≥ 10 copies of ie1) when 20 ng ($\sim 3,500$ cell equivalents) of DNA was subjected to PCR. Assuming that a mouse spleen contains approximately 10^8 nucleated cells, we

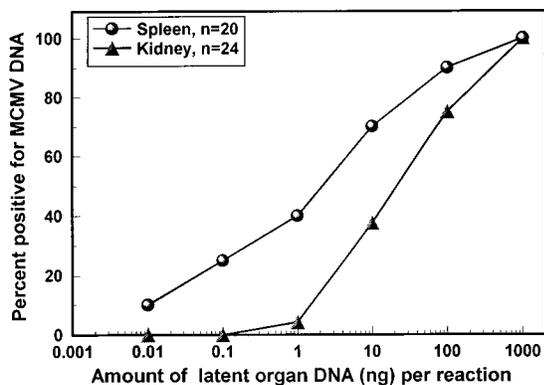


FIG. 5. Quantitation of the MCMV genome in latently infected organs. Spleen and kidney from latently infected mice were harvested, and DNA was extracted. One microgram of latent tissue DNA was used at the lowest dilution, and diluent contained naive liver DNA to keep total DNA constant in each reaction. DNA dilutions were entered into the nested PCR to detect the MCMV genome, and the product was visualized on an ethidium bromide-stained gel.

estimate that 10 copies of MCMV DNA per 350 cell equivalents corresponds to about 3×10^6 copies of the MCMV genome per spleen.

DISCUSSION

In this report, we show that spleens, kidneys, and salivary glands of mice allowed to recover from acute MCMV infection do not contain infectious MCMV by two separate sensitive detection assays. While infectious MCMV was not present, MCMV DNA was detected by nested PCR in both spleen and kidney. Quantitation of MCMV genomes in spleen and kidney revealed that there were an average of 3×10^6 copies of the MCMV genome per latently infected spleen, despite the fact that we did not detect infectious virus. The presence of latent MCMV was evidenced by reactivation from spleen and kidney fragments *in vitro* and by reactivation of MCMV from latent kidney cells adoptively transferred to SCID mice. These experiments demonstrate that MCMV latency does not require persistence.

Evidence for molecular latency. The distinction between a state of low-level persistence and molecular latency has relied heavily on the detection of infectious cytomegalovirus in tissues. Reported sensitivities of detection in published studies have ranged from 15 to 100 PFU in titer assays (2, 18, 29). Since we found that sonicated tissue inhibited detection of low levels of MCMV in plaque assays, we determined the sensitivity of two assays for detection of any infectious MCMV in latently infected tissues. We evaluated long-term culture with indicator monolayers since this assay had been reported to rule out persistence in latent MCMV tissues (2, 8). When we quantitated the sensitivity of this assay, we reproducibly detected 1 PFU of MCMV in appropriately diluted samples (Fig. 1 and 2). SCID mice were also sensitive to very low levels of MCMV and were used as a second independent assay. We evaluated latently infected spleen, kidney, and salivary gland tissues for presence of infectious virus and failed to detect any preformed infectious MCMV virions in 104 of 105 organs (Table 1). Quantitation of MCMV DNA in 48 latently infected organs indicated that organs maintain high levels of MCMV DNA months after infection (Fig. 5). This finding further argues that latency can exist without persistence since millions of copies of MCMV DNA remain in latently infected organs in which no infectious virus is detected.

Implications for analysis of *in vitro* reactivation and *in vivo* detection of immediate-early transcripts. In the absence of an assay capable of detecting a single PFU of MCMV, it has been difficult to distinguish between reactivation from latency and cultivation of persistent virus when tissue explants yield virus before day 10 of culture (18). Our data show, however, that true latency is the predominant form of virus infection in spleens 2 to 8 months after infection. Two groups have recently documented the presence of RNA from the MIE locus of MCMV in spleens (6) and lungs (29) of animals 2 to 12 months postinfection. In each case, investigators waited beyond the persistent stage in salivary glands before harvesting tissues. Both groups suggest that transcriptional activity is consistent with a low-level persistent infection which was not detected in culture assays. However, our data argue that the spleen, kidney, and salivary gland completely lack infectious MCMV after recovery from infection. Preliminary results with long term cocultures of latently infected lungs also indicate MCMV is not persistent at this site (unpublished data). This finding suggests either that MIE transcription is an integral part of the MCMV gene program during latency or that abortive reactivation is a common event resulting in the presence of MIE transcripts without production of progeny virus. Given the sensitivity of reverse transcriptase PCR, it will be critical for future studies of MCMV transcription during latency to include studies documenting the presence or absence of low-level persistence.

During clearance of infectious virus from visceral organs, there is likely a transitional stage in which persistence and molecular latency coexist. While we have shown that molecular latency can exist in the absence of persistent infection, it is still possible that intermittent reactivation with low-level persistence is necessary for long-term maintenance of the latent state. If this were the case, we would have expected to find that some but not all organs contain infectious MCMV after recovery from acute infection. We did find one kidney with 1 to 5 PFU of MCMV, but since only 1 of 105 organs tested contained virus, intermittent persistence is unlikely to be critical for latency.

Reactivation of latent tissues in SCID mice. We found infectious virus in salivary glands of 10 of 10 SCID mice on day 28 following an *i.p.* injection with 2×10^7 kidney cells from latently infected mice. We examined the contralateral kidney taken from the latent mice in this experiment and found no infectious virus in both *in vitro* and *in vivo* assays. Thus, this experiment demonstrates that SCID mice provide an environment in which reactivation from latency occurs. However, we have not been able to detect reactivation from spleen cells following transfer into SCID mice (unpublished data). It is possible that spleen cells simultaneously carry both a cell type from which MCMV can reactivate in cocultures with MEFs and MCMV-immune cells which either block reactivation or limit viral spread after reactivation *in vivo*. It will be interesting to use this model to dissect aspects of the immune system involved in maintenance of latency or prevention of viral spread after reactivation from latency.

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