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NOTES

Latent Murine Cytomegalovirus DNA in Splenic Stromal Cells of Mice

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Latency is an integral feature of the pathogenesis of cytomegalovirus infection and disease. Using in situ hybridization, we detected viral DNA in the splenic stroma of mice with acute murine cytomegalovirus (MCMV) infection but could not detect latent infection. By using enzymatic amplification of a 700-bp region of exon 4 of immediate-early gene 1 of MCMV, viral DNA was consistently detected in whole spleens of latently infected mice. MCMV DNA was detected in 16 of 23 stromal cell fractions from latently infected animals, in only 2 of 13 residual nonstromal cell fractions, and in none of 9 additional lymphocyte or macrophage-enriched nonstromal cell preparations. We conclude that MCMV DNA is maintained predominantly, and possibly exclusively, in stromal cells in the spleens of latently infected mice.

Cytomegalovirus (CMV) causes serious disease in utero (24) and in immunocompromised patients (7). The ability of CMV to establish a latent infection is an integral feature of the pathogenesis of CMV infection and disease (10). Despite extensive epidemiologic evidence that CMV can be transferred from donor to recipient by blood transfusion (29) and organ transplantation (3, 9), identification of the cell types which harbor the latent virus has been difficult.

The pathogenesis of latent murine CMV (MCMV) infection in mice has many similarities to latent human CMV infection. Thus, latent MCMV can be reactivated from its dormant state by immunosuppression (8, 12, 22) or transmitted from donor to recipient by organ grafts (16, 27) or blood transfusions (2). In terms of cell types which may be latently infected, the spleen has been studied most extensively. Here, the virus can be reactivated in the vast majority of animals by explantation of splenic tissue (11, 28) or, somewhat less efficiently, by cocultivation of spleen cells in suspension with permissive mouse embryo fibroblasts (13, 17–19). Recently, stromal cells from latently infected mouse spleens were implicated as the site of latent MCMV infection by cocultivation experiments (18). To date, however, latent MCMV has not been detected at the molecular level in the relevant cells. In this report, we demonstrate that latent MCMV DNA is maintained predominantly, and possibly exclusively, in stromal cells in the spleens of latently infected mice.

We first localized the site of replication of MCMV in the spleen during acute infection. Specific-pathogen-free BALB/c and C3H/HeN female mice were inoculated intraperitoneally with 10^5 PFU of salivary gland-passaged Smith strain MCMV, prepared as previously described (11, 12, 20). Four days later, the animals were sacrificed and spleens were harvested, fixed, and embedded in paraffin. In situ DNA-DNA hybridization was performed according to the methods of Haase et al. (5), with minor modifications. The *Eco*RI subgenomic MCMV DNA fragment A cloned in pACYC184 in *Escherichia coli* HB101 and provided by Deborah Spector (University of California, San Diego) was labeled with [^{125}I]dCTP and used as the probe. The results of a typical experiment are shown in Fig. 1. Virtually all of the MCMV DNA was detected in the perifollicular regions of the stromal splenic red pulp during the acute infection. No hybridization was detected in the splenic follicules (white pulp) in any animal.

After the acute MCMV infection resolved, we were not able to detect latent MCMV DNA in the spleens of either BALB/c or C3H mice by in situ hybridization. Hence, we used enzymatic amplification of MCMV DNA to search for latent infection. For these studies, latent infection was established by using previously described techniques (8, 11–13, 22, 23). Gene amplification was done as described by Saiki et al. (21), with minor modifications. We synthesized six sets of oligonucleotide primers (Fig. 2) that were selected from exon 4 of the MCMV immediate-early gene 1 from published sequence data (14). The 30-bp primer pair 566 and 567, which amplifies a 700-bp segment of MCMV, was used in most experiments. The base sequences are as follows: primer 566 (1701 to 1730), 5′-ATC-AAT-CAG-CCA-TCA-ACT-CTG-CTA-CCA-ACA-3′; and primer 567 (2400 to 2371 [antisense]), 5′-ATG-GTG-AAG-CTA-TCA-AAG-ATG-TGC-ATC-TCA-3′. Primers were separated on a polyacrylamide gel, eluted, dried, reconstituted in water, and quantified on the basis of optical density.

For gene amplification, DNA was prepared by a series of phenol-chloroform extractions, and 1 μg of sample DNA was added to a reaction mixture containing (final concentrations) 200 μM each deoxynucleoside triphosphate, 0.01% gelatin, 50 pM each primer, 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris HCl (pH 8.4) in a total volume of 100 μl. Reaction mixtures were incubated at 94°C for 10 min prior to the addition of 2.5 U of *Taq* (*Thermus aquaticus*) polymerase (Perkin Elmer-Cetus, Norwalk, Conn.). Each sample was

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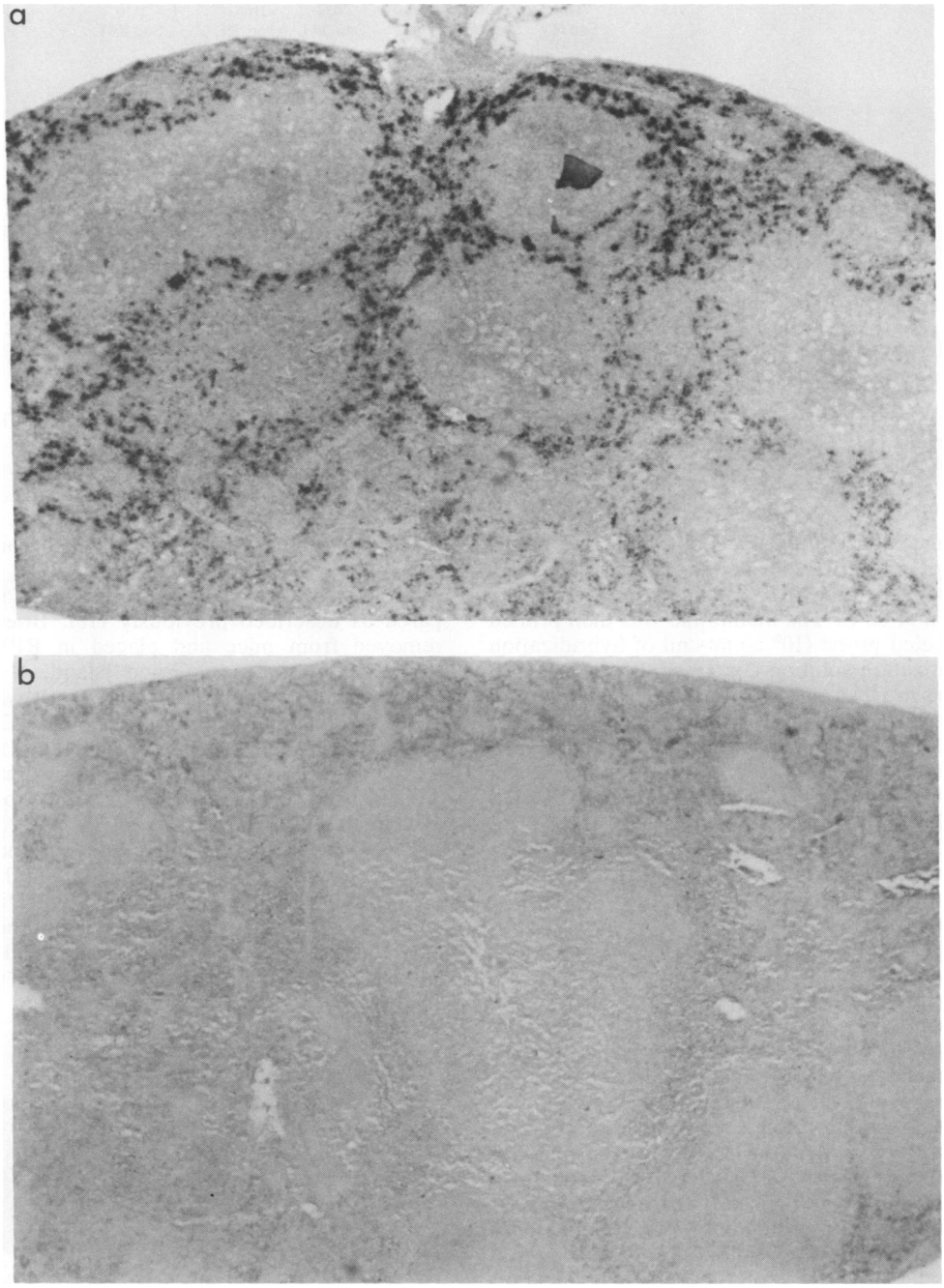

FIG. 1. (a) MCMV DNA was detected in the splenic stroma (red pulp) of a section of a spleen from a BALB/c mouse infected intraperitoneally 4 days earlier with 10^5 PFU units of MCMV. The slide was hybridized in situ with MCMV *Eco*RI subgenomic fragment A labeled with [^{125}I]dCTP. Treatment with DNase eliminated the signal (not shown). (b) No signal was detected in sections from an uninfected mouse.

amplified for 30 cycles in an automated thermal cycler (Perkin Elmer-Cetus). Each cycle entailed denaturation at 94°C for 1 min, annealing of extension primers at 68°C for 1.5 min, and primer extension at 72°C for 2 min, followed by a 10-min final extension at 72°C.

Amplified samples were electrophoresed on 1.2% agarose gels, stained with ethidium bromide, and photographed. Samples were then transferred to a nylon-backed membrane by electrophoretic transfer in 0.5% Tris-borate-EDTA (TBE), using a Trans-Blot Cell (Bio-Rad, Richmond, Calif.) apparatus.

A 30-bp oligonucleotide complementary to a sequence lying between the primer pairs was used as the probe and labeled with [^{32}P]dCTP by a tailing reaction using terminal deoxynucleotide transferase. Specific activity of the probe ranged from 10^9 to 10^{10} dpm/μg. Prehybridization was done in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.7 mg of salmon sperm DNA

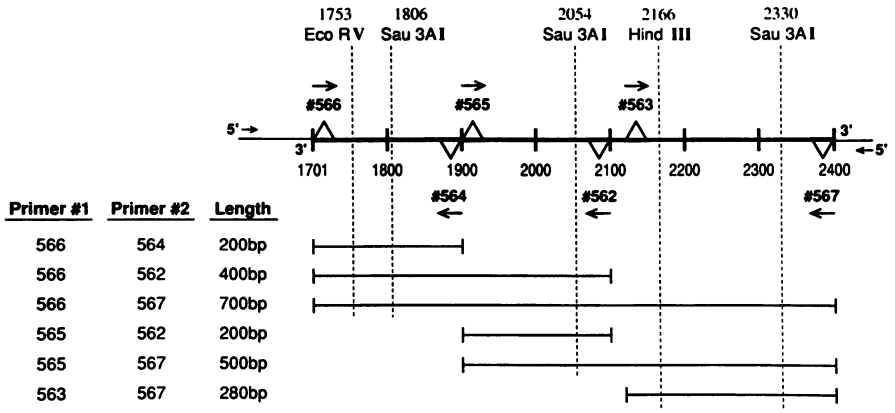

FIG. 2. Oligonucleotide primer pairs used for enzymatic amplification of the MCMV major immediate-early gene 1. The primer pairs flank DNA segments ranging in size from 200 to 700 bp.

per ml, 10× Denhardt's solution, 17 U of heparin per ml, 50 mM Tris HCl (pH 7.4), 0.2 mg of poly(C) per ml, 0.1% sodium pyrophosphate, and 0.1% sodium dodecyl sulfate (SDS) for 2 to 4 h at 68°C. The membrane was then probed with the radiolabeled probe (10^6 counts/ml of hybridization solution). Hybridization solution contained 2.5× SSC, 0.35 mg of salmon sperm DNA per ml, 5× Denhardt's solution, 8.5 U of heparin per ml, 25 mM Tris HCl, 0.1 mg of poly(C) per ml, 0.05% sodium pyrophosphate, and 0.05% SDS. The membrane was hybridized for 24 h at 68°C and washed at 55 to 68°C in 2× SSC–0.1% SDS for 15 min, 2× SSC–0.1% SDS for 30 min, and 0.1× SSC–0.1% SDS for 45 min. Hybridization was detected by autoradiography using XAR5 film (Eastman-Kodak Co., Rochester, N.Y.) and two intensifying screens (Cronex Lightning-Plus; DuPont de Nemours & Co., Newton, Conn.) at −70°C.

Positive control samples in each gene amplification experiment included standardized amounts of purified MCMV DNA and, in some experiments, DNA extracted from the spleen of a mouse with acute MCMV infection. Negative control samples included DNA from the spleens of uninfected mice, human leukocyte DNA, and samples containing polymerase chain reaction (PCR) reagents (including primers) but no DNA. In addition, physically separate pre- and post-PCR workstations, aliquots of premixed reagents, and positive displacement pipettes were used (26). Aliquots from each sample were studied in at least two separate experiments. Duplicate or multiple aliquots were counted only once if they came from the same animal.

To determine the sensitivity of the gene amplification procedure for MCMV, reconstruction experiments were performed in which known amounts of purified MCMV DNA were diluted in spleen cell DNA obtained from uninfected animals. The results of one such experiment are shown in Fig. 3. In all experiments, 20 fg of MCMV DNA was detected under these conditions. On some occasions, 10 fg was detected. These amounts of DNA correspond to approximately 40 to 80 copies of the MCMV genome.

In initial experiments, MCMV DNA was sought by enzymatic amplification of DNA extracted from whole spleens of latently infected and uninfected control BALB/c and C3H mice. MCMV DNA was amplified from the spleens of all of 13 latently infected mice and none of 9 uninfected, age-matched control animals. A representative Southern blot is shown in Fig. 4. There was no difference in the frequency with which latent viral DNA could be amplified from the spleens of BALB/c or C3H mice.

We next extracted DNA from splenic stromal cells prepared as described previously (18). Briefly, spleens were removed from mice and placed in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.). Spleen pulp was extruded from the capsule by gentle washing with RPMI 1640 medium through an 18-gauge needle. The pulp was then suspended in 12 ml of RPMI 1640 medium and transferred to a 15-ml conical centrifuge tube. The stromal fragments were defined as those fragments which settled to the bottom of the tube in 10 min (18).

MCMV DNA was detected in the stromal cells of 16 of 23 mice with latent MCMV infection and in the stromal cells of 1 of 23 uninfected mice by gene amplification ($P < 0.001$ by chi-square test). A representative Southern hybridization from this series of experiments is shown in Fig. 5. The DNA band detected in the sample from the one uninfected mouse

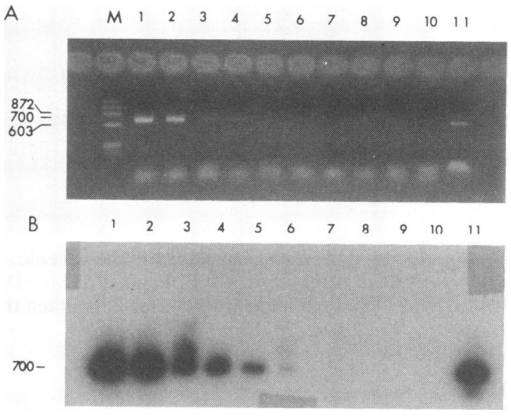

FIG. 3. Serial dilutions of MCMV DNA in 1 μg of murine genomic DNA amplified by PCR. (A) Agarose gel; (B) corresponding Southern blot autoradiograph. The sensitivity of the technique in this experiment was at least 76 copies of MCMV DNA detected in 1 μg of DNA. Lanes: 1 to 6, 10 pg, 5 pg, 1.25 pg, 313 fg, 78 fg, and 19 fg, respectively, of MCMV; 7 to 10, controls of reagents only, 1 μg of murine genomic DNA, 1 μg of human genomic DNA, and 2.5 pg of purified human CMV DNA, respectively; 11, 1 μg of spleen DNA from a mouse with acute MCMV infection.

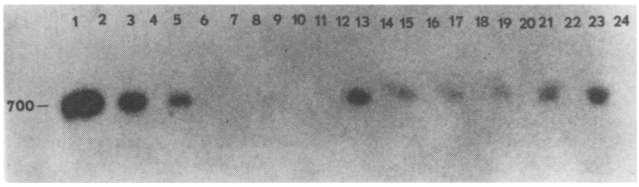

FIG. 4. MCMV DNA detected in spleens from BALB/c mice with latent MCMV infection. Shown is an autoradiograph of Southern hybridization of samples amplified by PCR. Lanes: 1, 3, 5, and 7, 1.25 pg, 312 fg, 78 fg, and 10 fg, respectively, of purified MCMV DNA reconstituted in 1 μg of murine genomic DNA; 9, 1 μg of murine genomic DNA; 11, reagents only; 2, 4, 6, 8, 10, and 12 not loaded; 13, 15, 17, 19, 21, and 23, 1 μg of DNA extracted from the spleens of six different latently infected animals; 14, 16, 18, 20, 22, and 24, 1 μg of DNA extracted from the spleens of six different uninfected mice.

was the only instance in which an apparent false-positive occurred among a total of 69 negative control samples in all of the experiments performed. We presume that this resulted from carryover of DNA from a positive control or sample. However, the possibility that this mouse was mislabeled cannot be excluded completely.

MCMV DNA was amplified from nonstromal spleen cells of 2 of 13 latently infected mice, while none was detected in nonstromal cells of 13 uninfected animals. The most likely explanation for the detection of MCMV DNA in the nonstromal cell fraction from these two mice is failure to remove all latently infected stromal cells from the suspension by the relatively crude sedimentation procedures used. If a small number of latently infected stromal cells remained among the nonstromal cells, MCMV DNA would have been detected by the sensitive enzymatic amplification procedure. To determine whether viral DNA in latently infected cells might be diluted because of the larger amount of cellular DNA in the nonstromal fraction, we further separated nonstromal spleen cells into macrophage-enriched (adherent) and lymphocyte-enriched (nonadherent) populations. No MCMV DNA could be amplified from the cellular DNA of either of these enriched nonstromal cell populations in experiments involving nine mice. Thus, overall we detected MCMV DNA in only 2 of 22 nonstromal cell fractions of spleens from mice with latent MCMV infection.

From the findings presented here, it appears unlikely that nonstromal cells in the spleen also harbor latent MCMV. Viral DNA was quite readily detected initially in whole

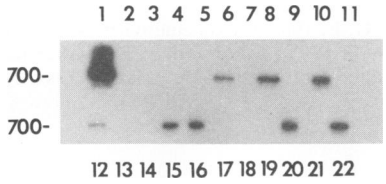

FIG. 5. Detection of MCMV DNA in the stromal fractions of spleens from C3H mice with latent MCMV infection. Shown is an autoradiograph of Southern hybridization of samples amplified by PCR. Lanes: 1, 1 pg of purified MCMV DNA; 2, PCR reagents only; 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21, 1 μg of DNA extracted from the stromal cell fractions of spleens from 10 different uninfected mice (the single false-positive sample referred to in the text is in lane 15); 4, 6, 8, 10, 12, 14, 16, 18, and 20, 1 μg of DNA extracted from the stromal cell fractions of spleens from 10 different mice with latent MCMV infection.

spleens and later in stromal cells from latently infected animals. In contrast, we were unable to detect latent MCMV DNA in the nonstromal cell fractions of spleens from the vast majority of latently infected mice. A possibility that cannot be excluded completely, however, is that a latently infected cell type is simply present less frequently in the nonstromal areas than it is in the stromal regions of the spleen. This possibility might also explain why MCMV cannot be reactivated by cocultivation of nonstromal cells (18). However, our results provide little support for a hypothesis that a population of latently infected spleen cells exists from which MCMV fails to reactivate in vitro.

In our studies and those of Mercer et al. (18), viral DNA was detected by in situ hybridization during acute but not latent MCMV infection. These findings suggest that the number of latently infected spleen cells or the number of copies of viral DNA per cell (or both) is extremely low or that the DNA may be present in a form which is not accessible to in situ hybridization. Here it should be noted that in the case of latent herpes simplex virus in neurons, viral DNA cannot be detected by in situ hybridization even though quantitative blot hybridizations indicate that the virus is present in amounts far exceeding the sensitivity of the technique (25).

We could detect approximately 10 to 20 fg of MCMV DNA under the experimental conditions described. Since a total of 1.0 μg of cellular DNA was probed in each experiment, our results indicate that a minimum of approximately 40 to 80 copies of the viral genome are maintained within approximately 10^5 stromal cells. We cannot estimate the maximum number of viral genome copies likely to be present from the experiments presented.

In earlier studies of Olding et al. (19) and Jordan et al. (11, 13), latent MCMV infection could be reactivated consistently when nonadherent spleen cells were cocultivated with permissive mouse embryo fibroblasts. Establishment of spleen explants from latently infected animals also resulted in release of infectious MCMV from nonadherent cells followed by lytic replication of virus in permissive adherent macrophages (11). Using the cell separation techniques available at the time, it was tentatively concluded that latent MCMV resided in lymphocytes, most likely in B cells (11, 19). However, in those experiments, splenic stromal cells would not have been depleted by the complement-mediated lysis techniques used to remove T cells and, in retrospect, were most likely the source of reactivated MCMV.

The precise identity of the cell type in the stromal fraction which harbors latent MCMV remains unknown. The splenic stroma, or perifollicular red pulp, is a ductal network of splenic sinuses supported by reticulum cells, fibroblasts, reticulin and collagen fibers, and numerous macrophages (6). The lumina of the splenic sinuses are lined by endothelial cells and sinusoidal-lining cells. In their ultrastructural studies, Mercer et al. determined that acute MCMV infection occurred predominantly in the sinusoidal lining cells, with virions being detected infrequently in endothelial cells and not at all in other splenic cell types (18). Although all the evidence available thus far could be interpreted to mean that the sinusoidal-lining cells support both the acute and latent MCMV infection, the possibility that other stromal cells harbor latent virus but do not support lytic viral replication has not been excluded. Definitive resolution of this issue will require the development of improved techniques for the identification and selection of the various cells present in the spleen now that procedures sensitive enough to detect latent MCMV DNA are available.

The spleen is not the only organ in the mouse which is latently infected with MCMV (1, 4, 15, 27). The virus can be transmitted from donor to recipient animals by implantation of latently infected cardiac (27) or renal (16) tissue as well as by transfusion of peripheral blood (2). The identification of stromal cells in the spleen as a reservoir of latent MCMV provides an attractive unifying hypothesis which could explain how transmission of the virus in humans occurs by transplantation of such diverse organs as the kidney, heart, and liver. It will be important to conduct similar studies of these organs to identify the cells which harbor latent MCMV. Further studies are also needed to determine the state of the viral genome and the extent of viral gene expression, if any, during latency.

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