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Biphasic Viremia and Viral Gene Expression in Leukocytes during Acute Cytomegalovirus Infection of Mice

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Circulating leukocytes are important in dissemination of cytomegalovirus (CMV) infection in humans. In the mouse model of murine CMV infection (MCMV), it has been shown that infection peaks on days 5 to 7 after experimental infection, when 0.01 to 0.1% of the circulating leukocytes contain viral DNA. In our laboratory, MCMV DNA was detected by in situ hybridization predominantly in the mononuclear cells on day 6 after acute infection. Infectious virus was recovered from day 6 mononuclear fractions in 16 of 16 mice compared with that from day 6 polymorphonuclear fractions in 4 of 16 mice. An eclipse phenomenon was noted in the blood leukocytes by quantitative blot hybridization: the amount of MCMV DNA present was small on day 2, diminished on days 3 and 4, and then increased markedly on days 5 and 6 in both the mononuclear and polymorphonuclear fractions immediately following viral augmentation in the liver and spleen. MCMV immediate-early and glycoprotein B (late) transcripts were present in pooled mononuclear fractions only on day 6 of acute infection but not in pooled polymorphonuclear fractions. Collectively, these data demonstrate that (i) circulating leukocytes, predominantly mononuclear, are involved in dissemination of MCMV; (ii) a primary viremia with dissemination of MCMV to reticuloendothelial organs (liver and spleen) occurs and is followed by viral amplification and a subsequent, more intense secondary viremia; and (iii) immediate-early viral mRNA and glycoprotein B mRNA transcripts are detectable only during peak infection on day 6 in mononuclear leukocytes but not in polymorphonuclear leukocytes.

Human cytomegalovirus (CMV) may frequently be recovered from the peripheral blood leukocytes of patients with disseminated infection (5, 9, 15, 33), and molecular studies have confirmed that human CMV infects the circulating leukocytes in humans (6, 10, 20, 26, 30). Murine CMV (MCMV) infection has been an excellent experimental model to study the interaction of CMV and various tissues or cells, including leukocytes (2, 14, 16, 23). There are numerous reports detailing the sequence and histopathologic consequences of organ involvement during acute MCMV infection of mice (14); however, little information is available concerning specific virus-cell interactions which occur during viremic dissemination prior to the infection of organs and establishment of viral latency. Mice infected with MCMV develop hematologic manifestations similar to those of human disease (2, 23). Bale and O'Neil (1) have demonstrated by in situ hybridization that viral DNA is present in 0.001 to 0.1% of circulating leukocytes in mice between days 5 to 7 after intraperitoneal inoculation of MCMV. When MCMV DNA-positive cells could be identified, they were usually mononuclear cells, possibly monocytes. MCMV could be recovered from unfractionated leukocytes of mice for approximately 3 weeks and was closely correlated with the detection of intracellular viral DNA. Experiments to determine whether MCMV was replicating in blood leukocytes were not performed. Here, we present our molecular analysis of acute MCMV viremia in mice. The results indicate that the viremia is quantitatively biphasic and that MCMV replication may be occurring in mononuclear cells.

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MATERIALS AND METHODS

MCMV stocks. MCMV (Smith strain) was maintained by serial passage in mice. Virus stocks were prepared from 10% salivary gland homogenates of mice infected 2 to 3 weeks earlier by intraperitoneal inoculation of 10^5 PFU of MCMV (17, 18).

Homogenates were prepared in Eagle's minimal essential medium and stored at −70°C after the addition of dimethyl sulfoxide at a 10% concentration. Quantitative assays of infectious virus were performed in secondary mouse embryo cells under overlay with 0.2% agarose. Assay cultures were maintained at 37°C for 5 days and then stained with crystal violet for enumeration of plaques under a dissecting microscope.

Infection of mice. Female BALB/c mice obtained from the National Cancer Institute (Bethesda, Md.) were used in all experiments. Animals were inoculated with virus at 7 weeks of age. The mice were injected intraperitoneally with a sublethal dose (10^5 PFU) of Smith strain MCMV.

Separation of leukocyte populations. Animals were sacrificed at various times. Blood was obtained by cardiac puncture and separated into mononuclear and polymorphonuclear fractions by using a Ficoll-Hypaque gradient (28).

Culture of peripheral blood leukocytes. To detect infectious MCMV, mouse embryo cell cultures were inoculated with 10^4 polymorphonuclear or mononuclear cells and observed for viral cytopathic effects for 10 days.

Detection of MCMV DNA by in situ hybridization. In situ DNA-DNA hybridization was performed according to the methods of Haase et al. (13) and Pomeroy et al. (24), with minor modifications, on enriched populations of mononuclear and polymorphonuclear leukocyte fractions (10^4 to 10^5 cells per slide). A 30-bp oligonucleotide probe homologous to sequences in exon 4 of the major immediate-early MCMV gene 1 was labeled with ^{35}S-dCTP by a tailing reaction employing terminal deoxynucleotide transferase (4, 24). The

base sequence of the probe (bases 1900 to 1871 [antisense]), was 5′-CCA-GAC-TCT-CTT-TTC-TGA-GGG-CCC-TAG-ATT-3′ (19). Specific activity of the probe ranged from 10^7 to 10^8 cpm/μg. DNA extracted from mouse embryo cells harvested 24 h after infection with MCMV (Smith strain) and uninfected mouse embryo cells were used as controls.

Quantitation of viral DNA. DNA was extracted from each leukocyte fraction and from pooled organs (liver, spleen, and salivary glands) by using phenol-chloroform and ethanol precipitation. DNA was quantified by optical density. Quantities of denatured DNA not exceeding 10 μg were spotted onto Duralon nylon membranes (Stratagene, La Jolla, Calif.) previously soaked in 6× SSC (1× SSC is 0.015 M NaCl plus 0.015 M sodium citrate) by using a microfiltration apparatus (Bio-Rad Laboratories, Richmond, Calif.) (28). The membrane was prehybridized at 50°C for 12 h and subsequently hybridized for 24 h with a probe (*Eco*RI A fragment of MCMV) radiolabeled by nick translation with [^{32}P]dCTP to a specific activity of 10^8 cpm/μg of DNA. The amount of viral DNA within samples of organ DNA was quantified by videodensitometry by employing a standard curve as previously described and expressed as picograms of MCMV DNA per 10 μg of cellular DNA (26–28).

Detection of MCMV immediate-early gene 1 mRNA. Polymorphonuclear and mononuclear leukocyte fractions were lysed in 0.5 ml of a solution containing 4 M guanidinium isothiocyanate, 0.1 M 2-mercaptoethanol, and 0.5% sarcosyl in 25 mM sodium acetate buffer (pH 7.0). Five micrograms of glycogen (Boehringer Mannheim, Indianapolis, Ind.) was added as a carrier, and extraction of RNA was performed according to the method of Chomczynski and Sacchi (3). Total RNA was precipitated from the aqueous phase by addition of an equal volume of isopropanol. The pellet was resuspended in 0.025 ml of sterile water treated with diethyl pyrocarbonate.

The immediate-early 1 gene of MCMV contains three introns. By using published sequence data, primers were selected to distinguish DNA from mRNA on the basis of the presence or absence of sequences from these introns (19, 24). Primer 814 is a 30-bp oligonucleotide from exon 2, and primer 567 is a 30-bp oligonucleotide from exon 4. The base sequences are 5′-TCA-CTT-GGA-TGA-GAA-CCG-TGT-CTA-CCC-ATC-3′ for primer 814 (bases 1200 to 1229) and 5′-ATG-GTG-AAG-CTA-TCA-AAG-ATG-TGC-ATC-TCA-3′ for primer 567 (base 2400 to 2371 [antisense]) (19). Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) and the oligonucleotide antisense primer 567 were used to initiate first-strand DNA synthesis of MCMV immediate-early gene 1 RNA transcribed from a region of exon 2 through exon 4 of the major MCMV immediate-early gene 1. The amplified DNA representing viral mRNA (985 bp) can be readily distinguished, on the basis of size, from the corresponding viral genomic DNA (1,200 bp) which contains two introns totalling 215 bp which are not transcribed. For each reaction, 1 μg of total RNA was used as the template.

MCMV cDNA generated by reverse transcriptase was amplified by PCR. Each reaction mixture contained 10 mM Tris-HCl (pH 8.4), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.5 μM (each) primers 567 and 814, 200 μM (each) deoxynucleoside triphosphates, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus) in a volume of 0.1 ml. Samples were overlaid with mineral oil. Each sample was amplified for 30 cycles in an automated thermal cycler (Perkin-Elmer Cetus) as follows: denaturation at 94°C for 1 min, annealing of extension primers at 55°C for 1.5 min, and primer extension at 72°C for 2 min, followed by a 10-min final extension at 72°C.

A 40-μl aliquot of each sample was electrophoresed on a 1.2% agarose gel, stained with ethidium bromide, and photographed. The cDNA was subsequently transferred to a nylon membrane (Duralon; Stratagene) by electrophoretic transfer with a Trans-Blot Cell (Bio-Rad) apparatus in 0.5% Tris-borate-EDTA (TBE). Southern hybridization was performed by using an appropriate oligonucleotide probe (564). The base pair sequence of probe 564 is (bases 1900 to 1871 [antisense]) 5′-CCA-GAC-TCT-CTT-TTC-TGA-GGG-CCC-TAG-ATT-3′ (19). The probe was labeled with [^{32}P]dCTP by a tailing reaction with terminal deoxynucleotide transferase (4, 24). The specific activity of the probe was 10^9 to 10^{10} cpm/μg. The probe was added to the hybridization solution containing the membrane, which was then hybridized for 24 h at 55°C. The 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% sodium dodecyl sulfate (SDS). The membrane was washed at 68°C in 0.1× SSC–0.1% SDS twice for 30 min each. Hybridization was detected by autoradiography.

Detection of glycoprotein B mRNA. The protocol for extraction of total RNA detailed above was followed for extraction of total RNA in experiments focused on the late glycoprotein B gene. An aliquot of the total RNA pellet was treated with DNase I to remove residual amounts of contaminating DNA. The pellet was resuspended in 0.1 ml of 40 mM Tris-HCl (pH 8.0)–10 mM NaCl–6 mM $MgCl_2$–0.01 mM dithiothreitol containing 10 U of RNase-free DNase I (Boehringer Mannheim) and 40 U of RNasin (Promega, Madison, Wis.) and incubated at 37°C overnight. DNase I was inactivated by the addition of 0.4 ml of the lysing solution described previously, and the RNA was precipitated with isopropanol. The pellet was resuspended in 0.025 ml of sterile water treated with DEPC.

Since the glycoprotein B gene of MCMV contains no introns, amplified DNA representing viral mRNA cannot be distinguished on the basis of size from corresponding viral genomic DNA. Two sets of oligonucleotide primers (external and internal) were selected from published sequence data of the glycoprotein B gene (25). The external primer pair (1137 and 1138) amplifies a 744-bp segment of the MCMV glycoprotein B gene, while the internal primer pair (1139 and 1140) amplifies a 408-bp segment. The base sequences are 5′-CAG-CCT-GGA-CGA-GAT-CAT-3′, primer 1137 (2379 to 2397); 5′-TCC-TCG-CAG-CGT-CTC-CAA-TC-3′, primer 1138 (3123 to 3103 [antisense]); 5′-CGT-GTA-TCT-CAT-CTT-CAC-GAG-3′, primer 1139 (2646 to 2667); and 5′-AGT-GTC-CAT-GTC-GGC-CGT-CA-3′, primer 1140 (3054 to 3034 [antisense]) (25).

MCMV mRNA was transcribed to cDNA with 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) according to the manufacturer's instructions, with 1 μg of the total RNA as a template. The 3′ primer (10 pmol) for the glycoprotein B mRNA transcript was primer 1138. MCMV cDNA generated by reverse transcription was amplified by a nested PCR. Each primary reaction mixture contained 10 mM Tris-HCl (pH 8.4), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.5 μM (each) primers 1137 and 1138, 200 μM (each) deoxynucleoside triphosphates, and 2.5 U of *Taq* DNA polymerase in a volume of 0.1 ml. Samples were overlaid with mineral oil and amplified in a DNA thermal cycler (Perkin-Elmer Cetus) for 30 cycles as follows: denaturation at 94°C for 1 min, annealing of extension primers at 55°C for 1.5 min, and primer extension at 72°C for 2 min followed by a 10-minute final extension at 72°C.

For the secondary amplification, 5 μl of the primary amplification product of each sample was added to a tube with 8 μl

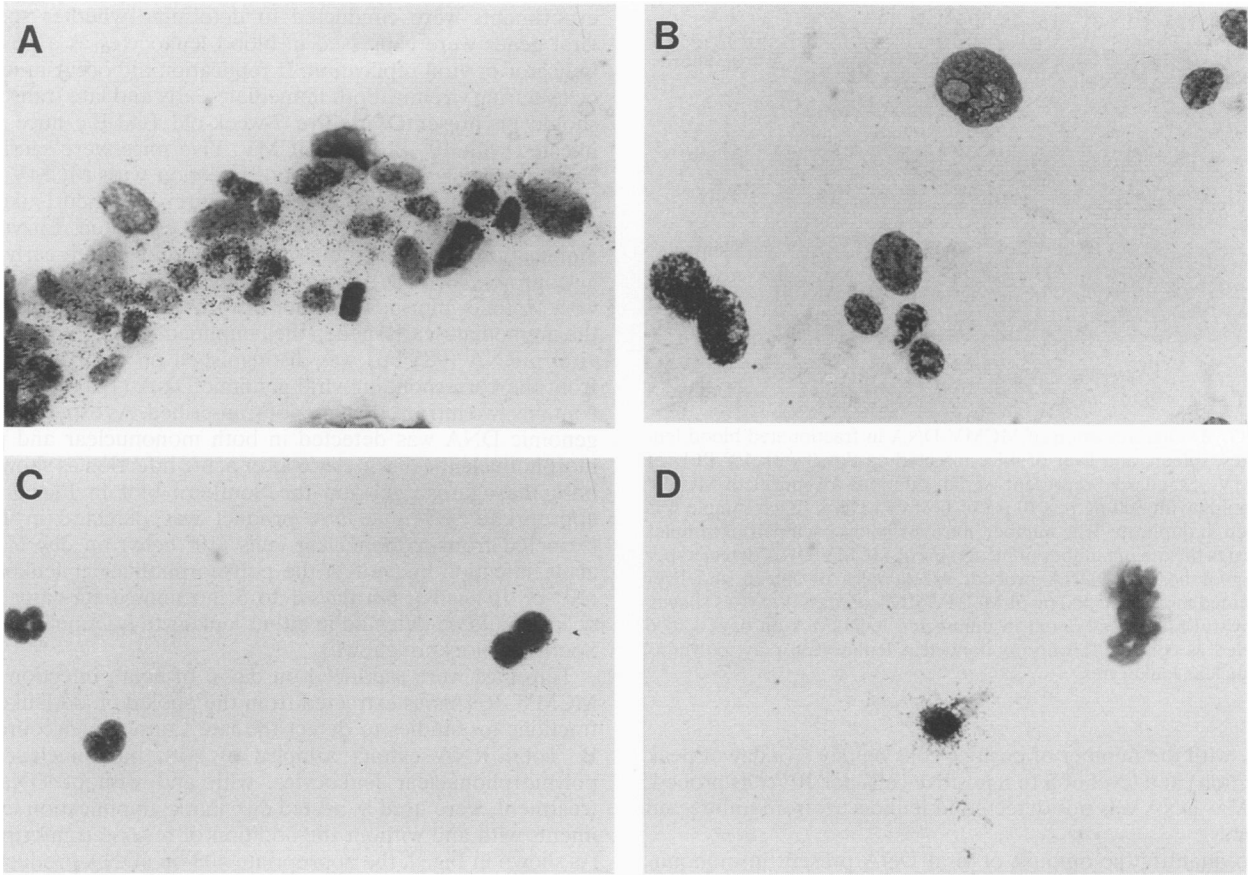

FIG. 1. Detection by in situ hybridization of MCMV DNA in mononuclear peripheral blood leukocytes of mice on day 6 of acute infection. A 30-bp oligonucleotide labeled with ^{35}S was used as the probe. (A) Mouse embryo cells infected with MCMV; (B) uninfected mouse embryo cells; (C) uninfected mononuclear leukocytes; (D) a mononuclear leukocyte from a mouse on day 6 of acute infection with detectable signal. Also shown is a cluster of polymorphonuclear leukocytes with no detectable signal. Magnification, ×100.

of water and 87 μl of a reaction mixture containing 2.5 μl of the 3′ internal primer (1140), 2.5 μl of the 5′ internal primer (1139), 0.5 μl of *Taq* DNA polymerase, 1.5 μl of each deoxynucleoside triphosphate, and 80 μl of 1× PCR buffer (containing 1.5 mM $MgCl_2$) overlaid with mineral oil. Each sample was then cycled an additional 30 times as described above. The nested reverse transcriptase PCR was performed on each sample with and without DNase incubation as described previously to detect the 408-bp PCR product amplified from cDNA of glycoprotein B mRNA. In addition, samples to be tested for the presence of MCMV RNA were studied with and without the addition of reverse transcriptase to verify that cDNA derived from the transcript and not viral genomic DNA had been detected.

Nested samples were analyzed as described above for the immediate-early gene 1 samples with electrophoresis, transfer to a nylon membrane, and Southern hybridization followed by autoradiography. A 21-bp oligonucleotide complementary to a sequence lying between the internal primer pairs was used as the probe. The base sequence of the probe is (bases 2796 to 2816) 5′-GCG-GCA-GTG-ACG-ATT-CGG-GTA-3′ (25).

Physically separate pre- and post-PCR stations were used, and barrier tip pipettes and aliquots of premixed PCR reagents were employed throughout. In each amplification experiment, positive control samples included total RNA extracted from mouse embryo fibroblasts infected with MCMV and total RNA extracted from the spleen of a mouse with acute MCMV infection. Negative controls, which were randomly interspersed among the samples, included total RNA extracted from uninfected mouse embryo fibroblasts and total RNA extracted from pooled polymononuclear and mononuclear leukocyte fractions of uninfected mice.

RESULTS

After inoculation of mice intraperitoneally with 10^5 PFU of MCMV, cultures of blood leukocytes yielded the virus on days 1 to 14 of acute infection. Since earlier investigators had found that infection peaked in the leukocytes on days 5 through 7, subsequent experiments were designed to focus on day 6. Sixteen 7-week-old BALB/c mice were injected intraperitoneally with MCMV and sacrificed on day 6 of infection. Blood was obtained by cardiac puncture and separated into mononuclear and polymorphonuclear fractions by Ficoll-Hypaque for each infected mouse (n = 16) and each uninfected control mouse (n = 3). Infectious virus was recovered from mononuclear cells in 16 of 16 mice and from polymorphonuclear cells in 4 of 16 mice on day 6 after infection. MCMV DNA was detected by in situ hybridization in mononuclear cell fractions from 14 of 16 mice but in polymorphonuclear cell fractions from only 2 of 16 mice on day 6. As exemplified in Fig. 1, MCMV DNA was detected almost exclusively in mononuclear

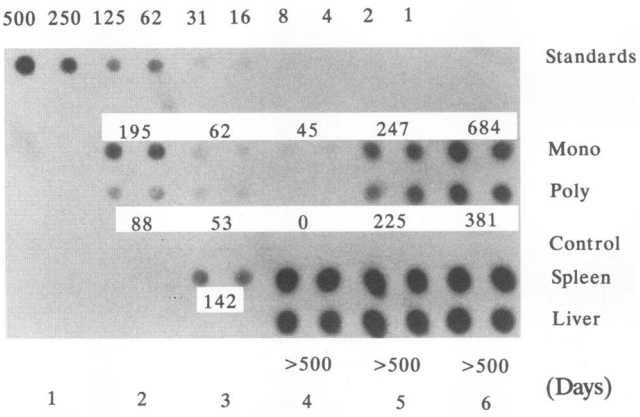

FIG. 2. Quantification of MCMV DNA in fractionated blood leukocytes, spleen, and liver of mice infected on day 0 with 10^5 PFU of MCMV. Standards represent serial dilutions of purified MCMV beginning with 500 pg per 10 μg of host cell DNA. Each sample was probed in duplicate. The number above or below each pair of samples indicates the mean number of picograms of MCMV DNA detected per 10 μg of host cell DNA probed. All samples of spleen and liver contained more than 500 pg of MCMV DNA on days 4 to 6, as shown. No hybridization was noted in uninfected leukocytes on days 1 to 6 (labeled as control). Mono, mononuclear leukocytes; poly, polymorphonuclear leukocytes.

cells, with the number of positive cells on day 6 (a day of peak infection) at a level of 5 to 8 positive cells per 10^4 cells probed. MCMV DNA was not detected in leukocytes from uninfected animals.

To quantify the amount of viral DNA present in mononuclear and polymorphonuclear leukocytes from initial infection until peak infection, blot hybridization with videodensitometry was utilized. Seven-week-old BALB/c mice were injected intraperitoneally with MCMV as described above. On days 1 to 6 of acute infection, four mice were sacrificed, and pooled blood, spleens, livers, and salivary glands were obtained. Pooled leukocyte specimens were also collected from uninfected animals to serve as negative controls. Serial dilutions of known quantities of purified MCMV DNA were used as standards. Relatively small amounts of MCMV DNA were detected in both leukocyte populations on days 2 and 3 after infection (Fig. 2). Viral DNA disappeared completely on day 4 in the granulocytes and declined markedly in the mononuclear cells as well. Subsequently, a much more intense secondary viremia recurred in both cell types on day 5, and the amount of MCMV DNA in mononuclear cells exceeded that in the neutrophils on day 6. During peak infection on day 6, 684 pg of MCMV DNA per 10 μg of cellular DNA was detected in mononuclear leukocytes, which corresponds to approximately 170 viral genomic equivalents per 100 cells. To determine whether the secondary viremia resulted from amplification of MCMV infection in the reticuloendothelial system, the amount of viral DNA also was quantified in the spleen and liver. MCMV DNA first appeared in the spleen on day 3 following infection, and large amounts were found in both the spleen and liver on day 4, at a time when leukocytes contained little viral DNA and just prior to the more intense secondary viremia (Fig. 2). Subsequently, a large amount of MCMV DNA remained in both organs. MCMV DNA was not detected in the salivary glands through day 6.

Because MCMV DNA was detected in both mononuclear and polymorphonuclear leukocytes during acute infection by two separate techniques (in situ and blot hybridizations), experiments were conducted to determine whether specific viral genes were expressed in blood leukocytes as a possible indicator of viral replication. If replication did occur in leukocytes during viremia, both immediate-early and late transcripts should be present. Fifty-five 7-week-old BALB/c mice were injected with 10^5 PFU of MCMV. Five mice were sacrificed each day for 6 days after acute infection with MCMV, and blood was obtained by cardiac puncture. Enriched leukocyte fractions were prepared, and RNA was extracted. Enzymatic amplications for detection of MCMV immediate-early and glycoprotein B mRNAs in each cell fraction were performed with Moloney murine leukemia virus reverse transcriptase. For the immediate-early gene, the amplified DNA representing viral mRNA (985 bp) was distinguished on the basis of size from the corresponding viral genomic DNA (1,200 bp) which contains two introns that are not transcribed. As expected, viral genomic DNA was detected in both mononuclear and polymorphonuclear cells on day 6 after acute infection as shown on both the agarose gel and the Southern blot in Fig. 3. The appropriate 985-bp cDNA product was detected in RNA extracted from mononuclear cells (10^6 cells) on day 6 after acute infection but not in the polymorphonuclear leukocytes (10^4 or 10^6 cells). On days 1 to 5, no immediate-early transcripts could be detected in either leukocyte fraction (gels and Southern blots not shown).

Ten mice were sacrificed on day 6 of acute infection with MCMV. RNA was extracted from the pooled blood leukocyte fractions for studies to detect the late transcript glycoprotein B. Total RNA extract samples of both mononuclear and polymorphonuclear leukocytes, with and without DNase I treatment, were used in nested enzymatic amplification experiments with and without the addition of reverse transcriptase. As shown in Fig. 4, the appropriate 408-bp cDNA product was detected in RNA extracted from mononuclear cells on day 6 after acute infection and treated with DNase I but not in the polymorphonuclear leukocytes. No cDNA was detected in samples in which reverse transcriptase was omitted from the reaction mixture.

DISCUSSION

In previous studies of viremia during acute MCMV infection of mice using quantitative assays of viral infectivity, extremely small numbers of cells have been found to carry the infectious virus (2. 32). In addition, mononuclear cells have been the main cell types involved. In the first molecular analysis of MCMV viremia, Bale and O'Neil (1) found that infection of leukocytes peaked on days 5 and 7 after inoculation of the virus, although the frequency of cellular infection reached only 0.01 to 0.1% of leukocytes. Our results are in accord with this level of quantitation and also support the initial observation in unfractioned leukocytes that mononuclear cells are primarily involved in dissemination of infection. Thus, infectious MCMV could be recovered from the mononuclear cells of all animals (16 of 16) at the time of peak viremia on day 6 and from only 4 of 16 polymorphonuclear cell preparations from the same animals. In addition, viral DNA could be detected consistently in a small percentage of mononuclear cells by in situ hybridization but only rarely in polymorphonuclear cells. Finally, the amount of MCMV DNA detected by quantitative blot hybridization was much higher in mononuclear cells on day 6 after infection than in the polymorphonuclear cells. At times, however, significant amounts of viral DNA were detected by the blot hybridization assay in the polymorphonuclear cells despite negative results by in situ hybridization and by viral culture. This apparent discrepancy could have resulted from the use of

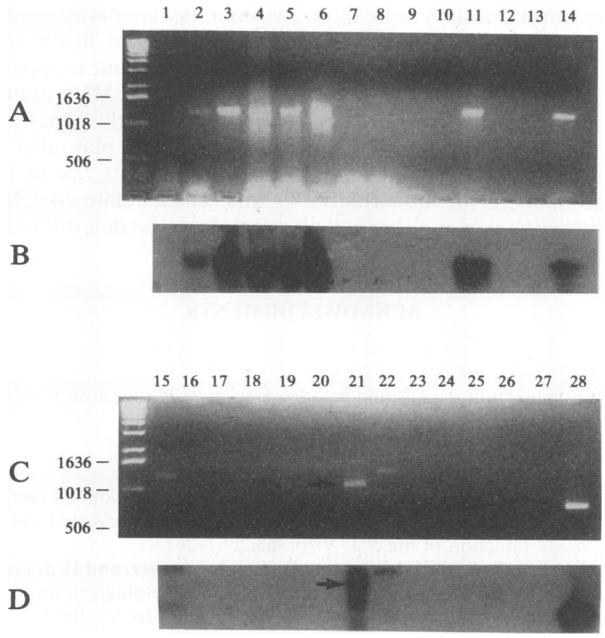

FIG. 3. Detection of mRNA transcript from a region of the major immediate-early 1 of MCMV in day 6 blood mononuclear cells of mice with viremia (lane 21). Agarose gels (A and C) are shown with the corresponding Southern blots (B and D, respectively) in a PCR experiment. Lanes: 1, RNA extracted from uninfected mouse embryo cells; 2 to 6, RNA extracted from mouse embryo cells infected with MCMV; 7 to 10, RNA extracted from uninfected mouse organs; 11, RNA extracted from acutely infected spleen; 12, 13, 18, and 23, RNA extracted from unfractionated leukocytes of uninfected mice (10^4 cells); 14, 15, and 26, RNA extracted from mononuclear leukocytes of acutely infected mice on day 6 (10^4 cells); 19, 20, and 27, RNA extracted from polymorphonuclear leukocytes of acutely infected mice on day 6 (10^4 cells); 16 and 24, RNA extracted from mononuclear leukocytes of uninfected mice (10^6 cells); 17 and 25, RNA extracted from polymorphonuclear leukocytes of uninfected mice (10^6 cells); 21, RNA extracted from mononuclear leukocytes of acutely infected mice on day 6 (10^6 cells); 22, RNA extracted from polymorphonuclear cells of acutely infected mice on day 6 (10^6 cells); 28, MCMV DNA PCR with primers to produce a 700-bp product. The smaller 985-bp mRNA transcript detected as cDNA in lane 21 (arrows) in mononuclear cells was not present in the polymorphonuclear cells (lane 22). Residual viral genomic DNA (1,200 bp) remaining after guanidinium thiocyanate extraction was detected in both mononuclear (lanes 14, 15, and 21) and polymorphonuclear (lane 22) cells. Molecular weight markers are indicated on the left.

a much smaller oligonucleotide probe in the in situ hybridization assays compared with the larger (32-kb) subgenomic *Eco*RI fragment used for blot hybridization. This explanation seems unlikely, however, because Bale and O'Neil used the 34-kb *Hin*dIII A fragment in their in situ hybridization experiments and were unable to detect MCMV DNA in polymorphonuclear cells. Another possibility is that viral DNA was present in large amounts in a very rare polymorphonuclear cell (or in contaminating mononuclear cells) that was not detected by in situ hybridization. Finally, phagocytic degradation of MCMV DNA could have occurred in the polymorphonuclear cells, resulting in inactivation of infectious virus but persistence of DNA fragments that were detected only by the larger subgenomic probe. In summary, our results do not exclude the possibility that neutrophils play some, albeit minor, role in MCMV viremia.

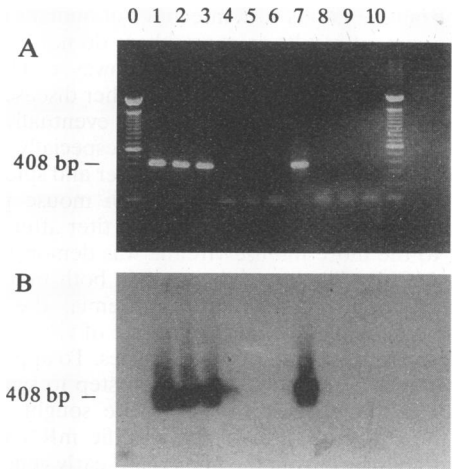

FIG. 4. Detection of mRNA transcript from a region of glycoprotein B (late) gene of MCMV. (A) Agarose gel; (B) Southern blot. Lanes: 0, DNA molecular weight markers; 1, RNA extracted from MCMV-infected mouse embryo cells; 2, RNA extracted from spleen of a mouse acutely infected with MCMV; 3, RNA extracted from day 6 mononuclear cells (10^6 cells) of acutely infected mice and incubated with reverse transcriptase; 4, RNA extracted from day 6 mononuclear leukocytes (10^6 cells) of acutely infected mice without reverse transcriptase incubation; 5 and 6, RNA extracted from day 6 polymorphonuclear leukocytes (10^6 cells) of acutely infected mice with and without reverse transcriptase incubation, respectively; 7 and 8, RNA extracted from day 6 mononuclear leukocytes (10^6 cells) of acutely infected mice treated with DNase I with and without reverse transcriptase incubation, respectively; 9 and 10, RNA extracted from day 6 polymorphonuclear leukocytes (10^6 cells) of acutely infected mice treated with DNase I with and without reverse transcriptase incubation, respectively.

A previously unrecognized biphasic aspect of MCMV viremia was clearly documented in these experiments. By a quantitative DNA blot hybridization assay, no MCMV DNA was detected in either mononuclear or polymorphonuclear cells 24 h after intraperitoneal infection. Forty-eight hours after infection, viral DNA was detected in both cell populations, when the amount of MCMV DNA in mononuclear cells was approximately twice that in the granulocytes. On the third and fourth days after infection, the amount of MCMV DNA declined markedly in both cell types and disappeared completely in the polymorphonuclear cells on day 4. On day 5, however, viral DNA returned in both cell populations at a high level and increased even further during peak infection on day 6, especially in the mononuclear cells. This more intense secondary viremia clearly followed the detection of MCMV infection in the spleen on day 3 and a marked amplification of viral DNA in both the spleen and liver on day 4. The findings are consistent with the interpretation that the secondary viremia resulted from replication and release of infectious virus from cells within these reticuloendothelial organs and recall Fenner's classic experiments in animals infected with infectious ectromelia virus of mice (mouse pox virus) (7, 8). In the latter experiments, inoculation of mouse pox virus into the footpad or ear skin of mice was followed by spread to the regional lymph nodes and, then, via a low-grade viremia, to the liver and spleen. Subsequent necrosis of infected cells within these organs was associated with an increase in the viral titer in blood and the eventual development of characteristic skin lesions. This sequence of events has been thought to be an integral component of the pathogenesis of varicella and pos-

sibly other exanthematous viral infections of humans (12). The MCMV experiment results presented here do not parallel the results of Fenner's studies completely, however. The mouse pox viremia was not biphasic per se but rather disease resulted from a gradually increasing viremia that eventually became intense enough to produce skin lesions, especially after the viral infection had been amplified in the liver and spleen (7, 8). However, no phase of infection in which mouse pox virus disappeared from the blood or declined in titer after an initial peak prior to the more intense viremia was demonstrated.

Because MCMV DNA was detected in both mononuclear and polymorphonuclear cells during viremia, the question arose whether gene expression or evidence of viral replication could be detected in either or both cell types. To approach this question, we used a reverse transcription step to synthesize a cDNA copy of the specific mRNA to be sought and then amplified the cDNA by PCR. The specific mRNAs sought were those transcribed from the immediate-early gene 1, which is transcribed prior to viral DNA synthesis, and the late glycoprotein, which is transcribed after viral DNA synthesis and produces a major viral structural protein (19, 25). Thus, detection of both viral transcripts would imply that complete viral replication is likely to have occurred whereas detection of early immediate-early 1 transcripts would indicate that the viral replicative cycle is blocked or that an abortive infection has occurred. In the experiments described, both MCMV mRNAs were detected in the mononuclear leukocytes at the time of peak infection on day 6 but not in the preceding days prior to maximal augmentation of the secondary viremia. These results suggest that viral replication in these cells may be dependent upon a relatively high multiplicity of infection and that lower multiplicities of infection are insufficient to produce either partial or complete viral replication. Although infection of mononuclear cells is certainly important for dissemination of MCMV throughout the tissues, whether replication of the virus per se within these cells makes a significant additional contribution to the pathogenesis of infection cannot be determined from these experiments. However, viral replication within mononuclear cells could theoretically result in delivery of larger amounts of infectious virus to the tissues. Interestingly, we were unable to detect either viral transcript in polymorphonuclear cells even when the amount of viral DNA with the granulocytes approached that detected in mononuclear cells during the peak viremia. This observation provides further evidence that the mononuclear cells play a much greater role in the pathogenesis of MCMV dissemination than granulocytes as noted above. Previously, investigators have reported mononuclear leukocytes may also harbor latent MCMV (2, 17, 21, 29).

In some respects, cytomegalovirus viremia in mice is similar to that in humans although there are some significant differences, perhaps attributable to the predominance of mononuclear leukocytes in the peripheral blood of mice. Quantitatively, the amount of viral DNA within leukocytes is surprisingly similar although in humans polymorphonuclear cells contain significantly greater amounts than do mononuclear cells (26). Thus, the median amounts of viral DNA in granulocytes and mononuclear cells in our studies of patients with AIDS who had visceral organ disease due to CMV were 632 and 325 pg per 10 μg of cellular DNA, respectively (27). In addition, in virtually all studies of human CMV viremia, the virus is much more frequently recovered from granulocytes than from mononuclear cells (5, 15, 26), although both cell types are clearly involved in viral dissemination (6, 10, 11, 31). With regard to viral gene expression, both the immediate-early genes and an early gene (pp65) appear to be expressed in polymorphonuclear cells during viremic dissemination in humans (6, 31). Whether late genes are expressed in infected granulocytes has not been resolved definitively, but it appears unlikely. In mononuclear cells of humans with CMV viremia, recent studies indicate that the immediate-early genes are virtually always expressed and that late genes may also be transcribed in some patients (10, 22). Whether, as in the mouse, there are quantitative factors which relate to CMV gene expression in either cell type remains to be determined in humans.

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