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Cytomegalovirus (CMV), as do other herpesviruses, establishes a lifelong latent infection in its natural host. While in immunologically intact hosts most CMV infections are subclinical, clinical disease follows severe immunosuppression and immunodeficiency. In these situations CMV may produce serious life-threatening disease, and virus reactivated from the latent state is often responsible. Essential to understanding this virus and its pathogenesis is the need to define particular tissue and cell types harboring viral DNA. We searched for viral DNA and RNA in subpopulations of blood cells from mice latently infected with murine CMV by using differential centrifugation and fluorescent antibody cell sorting followed by polymerase chain reaction analysis. Following intravenous inoculation, the viral DNA was found to be present in the buffy coat at and after 21 days postinfection, and both granulocytes and peripheral blood mononuclear leukocytes (PBML) were reservoirs. Further analysis of the PBML fraction by separation into Mac-1^+ and Mac-1^- cells revealed that monocytes harbored the DNA while lymphocytes were not sites of persistence. We conclude that in buffy coat of latently infected mice the viral DNA is present only in cells of the myeloid lineage. The relationship of this DNA to the latent infection is discussed.

INTRODUCTION

Persistent viral infections, which include both latent and chronic active infections, are common, and cytomegalovirus (CMV) is one such infection receiving attention because of its relationship to immunosuppressive states. Although most CMV infections are subclinical, clinical disease is frequently associated with severe immunosuppression, especially in settings of allograft transplantation (Fiala *et al.,* 1975; Smyth *et al.,* 1991), multiple blood transfusions (Adler, 1983), immunosuppressive drug treatment of patients with terminal leukemias (Mayo and Rapp, 1980) and lymphomas (Duwall *et al.,* 1966), and more recently in those with acquired immunodeficiency syndrome (Jacobson and Mills, 1988; Hirsch, 1991). In these immunosuppressed and immunocompromised individuals, CMV may result in serious life-threatening disease and in many, if not most, instances the disease results from replication and dissemination of a persistent virus, which may have previously been latent.

Since "reactivated" CMV is a significant cause of disease, an understanding of the latent state is essential in development of any strategy to prevent or control the process. A central issue is the need to define the particular tissue and cell types in which the viral DNA persists. With respect to the blood/vascular system, correlation between peripheral blood transfusion and human CMV (HCMV) transmission has been long noted (Adler, 1983; Cheung and Lang, 1977; Winston *et al.,* 1980). In more defining studies, peripheral blood mononuclear leukocytes (PBML) have been extensively evaluated and are suspected both of disseminating virus and of harboring the latent viral genome (Bale and O'Neil, 1989; Brautigam *et al.,* 1979; Collins *et al.,* 1994; Jordan and Mar, 1982; Schrier *et al.,* 1985; Stannier *et al.,* 1989; Stoddart *et al.,* 1994; Taylor-Wiedman *et al.,* 1991). However, discrepancies exist between the various studies regarding the particular subsets of cells involved. In addition, while the PBML fraction of blood has been the object of considerable study, analysis of polymorphonuclear leukocytes (PBML) for CMV persistence has received only limited attention (Taylor-Wiedeman *et al.,* 1993). HCMV has been detected during acute infection in the PMNL fraction of peripheral blood of both immunocompetent and immunocompromised patients (Rinaldo *et al.,* 1977; Saltzman *et al.,* 1988; Dankner *et al.,* 1990; Gerna *et al.,* 1991; Revello *et al.,* 1992), and murine CMV (MCMV) and MCMV DNA have been detected in the PMNL fractions during acute infections of mice (Collins *et al.,* 1994). There is also clear evidence of viral gene expression in PMNL since both immediate early and pp65 viral proteins of HCMV are expressed in these cells during virema (Gerna *et al.,*

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1991; Revello *et al.,* 1992). In addition, persistent CMV DNA has been detected in myeloid bone marrow cells that serve as progenitors for both peripheral blood granulocytes and monocytes (Kondo *et al.,* 1994). Although these results suggest that blood PMNL and their precursors may undergo acute and latent CMV infection, results from one study of persistent HCMV (Taylor-Wiedeman *et al.,* 1993) indicated that PMNL do not harbor HCMV DNA.

The focus of the work presented here was the systematic identification of the cell types in blood in which the CMV genome persists in the latently infected hosts. For ease in experimental manipulation, and since the system appears to closely resemble its human counterpart, we used the murine virus (MCMV) in its natural host. The fact that virus would still be replicating in some tissues 3 weeks postinfection has been well documented in the literature and this includes work by our group. As a matter of fact, since we have recently shown that persistent MCMV exists simultaneously in both chronic active and latent states and that the chronic active infection in the lung may persist indefinitely (Yuhasz *et al.,* 1994), it is unlikely that the virus is ever completely latent in an infected animal. However, we found here that by 21 days postinfection viral RNA could no longer be detected, indicating the resolution of acute infection in the blood. For the purpose of discussion, this is the condition to which we refer as latently infected even though the virus may not be latent at other anatomical locations. Using differential centrifugation followed by quantitative PCR analysis, we found that both PBML and PMNL harbored the persistent MCMV genome. Additional subfractionation of the PBML fraction into monocytes and lymphocytes by fluorescent antibody cell sorting (FACS) revealed that MCMV DNA was detectable only in the monocyte fraction; lymphocytes were not involved. Since monocytes and PMNL are both phagocytic scavengers, the possibility that the DNA detected was due to phagocytosis of virus or viral DNA which is not maintained latently is suggested. Although this possibility could not be ruled out, our reasons for favoring a latent infection of these cells are discussed.

MATERIALS AND METHODS

Tissue culture and virus strains

Primary mouse embryo fibroblasts (MEF) used for viral titrations and production of viral RNA were prepared from 18- to 20-day gestation BALB/c ByJ mouse embryos (Jackson Laboratories, Bar Harbor, ME) and maintained as previously described (Thompson and Stevens, 1983). The Smith strain of MCMV (American Type Culture Collection, Rockville, MD) used in these studies was maintained by serial passage in mice (Jordan *et al.,* 1977). In general, female BALB/c ByJ mice 6–8 weeks of age were infected by tail vein injection and viral stocks prepared as a 10% (w/v) homogenate of submandibular salivary gland tissue in Eagle's minimal essential medium (MEM) 12 days p.i.

Infection of mice, collection of blood, and titration of virus

For both the experimental animals and the production of viral stocks, female BALB/c ByJ mice 6–8 weeks of age were infected by injection into the tail vein with $1–5 \times 10^4$ plaque-forming units (PFU) of MCMV, which was sufficient to kill approximately 10% of the animals. Blood was collected from mice by bleeding from the axillary plexus (Donovan and Brown, 1991) and treated with 0.1 ml of heparin (1000 units/ml in ddH_2O; Sigma, St. Louis, MO). The whole-blood samples and the subsequent fractions were quantitated by hemacytometer counts and also analyzed qualitatively for both viability and purity. Cell viability was determined using trypan blue exclusion staining while purity was determined by microscopic differential counts of Giemsa-stained smears. Whole blood from 29 mice was evaluated for quantity and quality. Viral stocks were titrated on MEF monolayers with an overlay of MEM plus 10% fetal calf serum plus 0.2% agarose (Henson *et al.,* 1966; Shanley *et al.,* 1979).

Gradient fractionation of cell populations

Preparation of buffy coat containing all leukocyte subpopulations was performed by layering heparinized whole blood obtained from individual mice onto 5-ml gradients of Ficoll–Paque (Pharmacia). The loaded gradients were allowed to sit undisturbed for 90 min at room temperature and then the entire gradient down to, but excluding, the erythrocyte pellet was collected into fresh tubes. The Ficoll–Paque was diluted by adjusting the sample volume to 15 ml with sterile PBS and mixed thoroughly, and buffy-coat cells were pelleted by centrifugation at 450 *g,* 8 min, 18–20°. Contaminating erythrocytes were removed by a single treatment of 0.83% NH_4Cl and the cells were washed twice with MEM. The final pellets from 13 animals were quantitatively and qualitatively analyzed. Other samples were further fractionated by additional differential centrifugation or fluorescent-activated cell sorting, or frozen and stored at −70° for PCR analysis. The buffy coat prepared from whole blood [$5.1 \times 10^6 \pm 2.5$ (±SD) nucleated cells/mouse] yielded $2.2 \times 10^6 \pm 1.6$ cells with approximately equal numbers of PMNL ($44 \pm 11\%$) and PBML ($56 \pm 11\%$).

Buffy coat prepared as just described was separated into PMNL and PBML fractions using a second Ficoll–Paque gradient. These gradient separations were performed essentially as described by the manufacturer and the mononuclear cells were recovered from the interface while the PMNL were isolated by diluting the remaining gradient and pelleting the cells as described for the buffy coat preparation. Both fractions were washed with MEM

as described above. This protocol provided $1.1 \times 10^5 \pm 9.4$ PBML with a 94 ± 4% purity and $2.3 \times 10^5 \pm 2.6$ PMNL with a purity of 86 ± 5% (10 samples quantitated and qualitated).

An alternative method for recovery of PBML involved loading whole blood onto Ficoll–Paque gradients and processing as described by the manufacturer. In general, heparinized whole blood was diluted with PBS and loaded onto 5-ml gradients of Ficoll–Paque and centrifuged (450 *g,* 3 min, 20°). The cell band at the interface was collected and washed as described above for the isolation of PMNL and PBML fractions. When this alternative procedure was used, the PBML at the interface were the only cells collected and yielded $5.6 \times 10^5 \pm 4.9$ cells with a 99.6 ± 0.2% purity (six samples quantitated and qualitated).

Fluorescent-activated cell sorting

Lymphocyte–monocyte-enriched samples were obtained using the alternative method for recovery of PBML as described above, pooled (three mice/sample), and sorted by FACS on a FACScan Plus (Becton–Dickinson, Mountain View, CA) made available through the University of California, Los Angeles, Flow Cytometery Core Facility Laboratory. FITC-conjugated anti-mouse Mac-1 (rat IgG_{2b} anti-CD11b) mAb (PharMingen, San Diego, CA) was diluted 1:100 and used to stain the PBML cells (Mitchell and Stevens, 1996). The specificity of binding of the Ab was controlled with a FITC-conjugated rat IgG_{2b} isotype control (1:50; PharMingen).

DNA and RNA extraction

Extraction of both DNA and RNA from individual samples of buffy coat and subsequent fractions was performed using TRIzol Reagent (Gibco BRL, Gaithersburg, MD) as suggested by the manufacturer except as noted below. The DNA pellets were incubated 5 min, 56°; 30 min, 37° in 8 m*M* NaOH to facilitate resuspension of the DNA, and the final DNA samples were extracted once with phenol:chloroform:isoamyl alcohol (25:24:1). DNA and RNA were precipitated with ethanol and sodium chloride using glycogen as carrier. The final DNA and RNA samples were resuspended in ddH_2O.

Reverse transcription and polymerase chain reactions

RNA samples were reverse transcribed to produce cDNA templates for RT-PCR amplification as previously described (Yuhasz *et al.,* 1994) using $pd(N)_6$ primers. Primers used for amplification of the MCMV immediate early-1 (ie-1) gene and the mouse adenine phosphoribosyltransferase (APRT) gene have been previously described (Yuhasz *et al.,* 1994). For ie-1 DNA PCR, 20% of the extracted DNA from each sample analyzed was incubated in a 50-μl reaction containing 67 m*M* Tris–HCl, pH 8.3; 16.6 m*M* $(NH_4)_2SO_4$; 20–22 pmol of each of the primers; 185 μg/ml bovine serum albumin; 1 μCi [^{32}P]dATP; 250 μ*M* each cold deoxynucleotide; 2.5 units *Taq* polymerase (Perkin–Elmer Cetus, Norwalk, CT) and 5 m*M* $MgCl_2$. The samples were processed in a thermal cycler (Ericomp, Inc., San Diego, CA). The first cycle consisted of 3 min each at 94, 68, and 72° followed by 30 cycles at the same temperatures for 1 min each. The sample volume was adjusted to 100 μl with TE buffer, extracted with chloroform:isoamyl alcohol (24:1), and precipitated. Thirty percent of the product was electrophoresed on a nondenaturing 5% polyacrylamide gel (29:1 acrylamide:bis-acrylamide) and the gel was dried and exposed to X-ray film with intensifying screens.

For both ie-1 and APRT RT-PCR, one-tenth (10 μl) of the final reverse transcription reaction mixture was used as template as described for the ie-1 DNA except 2.5 m*M* $MgCl_2$ was used for the APRT RT-PCR along with 1 cycle of 3 min each at 94, 55, and 72° followed by 30 cycles at the same temperatures for 1 min each. APRT DNA was amplified by PCR as described for ie-1 DNA except 2.5 m*M* $MgCl_2$ was used.

The first DNA PCR or RT-PCR amplification of samples analyzed for ie-1 DNA or RNA by double PCR was as described above except the radiolabeled nucleotide was omitted. Following chloroform:isoamyl alcohol extraction, 10% of the product of the first reaction (10 μl) was added to a second reaction including the radiolabeled nucleotide. Confirmation of PCR product specificity by Southern blotting for the ie-1 primers has been previously described (Yuhasz *et al.,* 1994).

Autoradiographs of PCRs were scanned using TWAIN technology with a Logitech ScanMan Color scanner and FotoTouch color imaging editing software, Version 1.1 (Logitech, Inc., Fremont, CA). The scanned images were analyzed with SigmaGel gel analysis software (Jandel Corp., San Rafael, CA) and compared as previously described (Yuhasz *et al.,* 1994) to standard curves of amplified plasmid DNA containing the MCMV *Eco*RI fragment E (Mercer *et al.,* 1983) or amplified cDNA from RNA produced according to the manufacturer's directions (Stratagene, La Jolla, CA) from a Bluescript plasmid containing the MCMV *Hin*dIII L fragment (Mercer *et al.,* 1983).

RESULTS

Detection of MCMV ie-1 DNA and RNA in buffy coat of mice acutely infected

To assess our ability to detect MCMV DNA and RNA in samples, blood was collected from infected and mock-infected mice 3 days p.i. and the DNA and RNA were separately extracted from samples obtained from individual mice. The DNA and RNA samples from three mice/group in each of three independent experiments were analyzed by PCR and RT-PCR, respectively. The primers

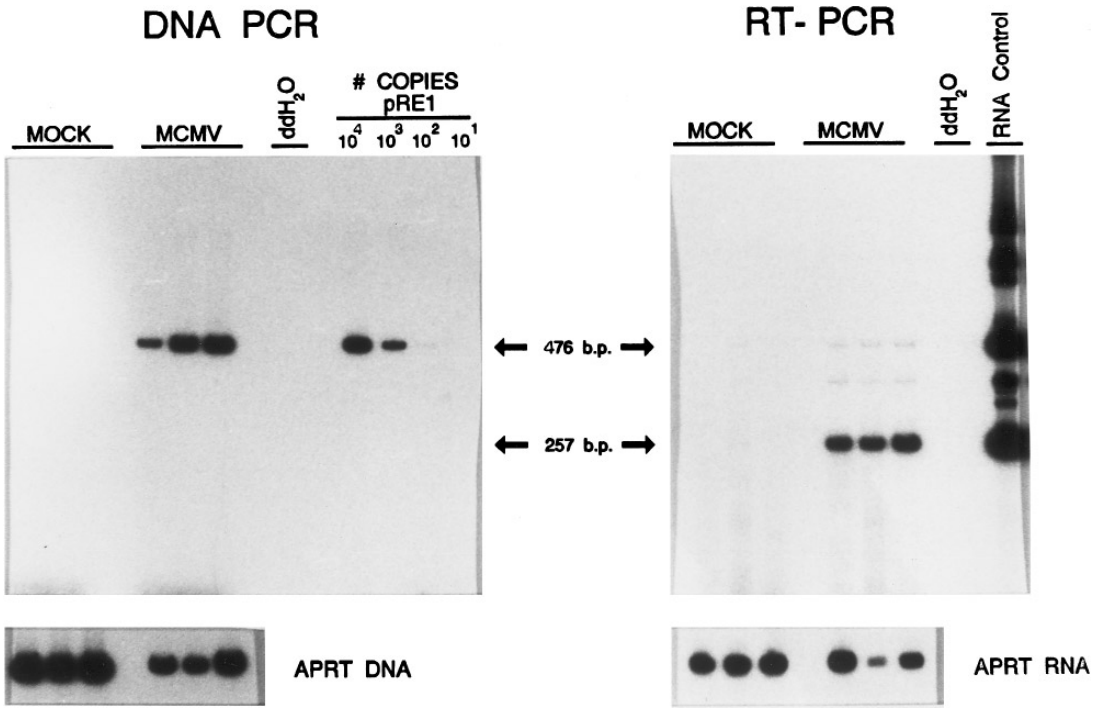

FIG. 1. MCMV ie-1 DNA and RNA in buffy coat of acutely infected mice. Buffy-coat cells were collected from individual mock-infected mice and mice infected for 3 days with MCMV. The DNA and RNA were extracted and analyzed by PCR and RT-PCR, respectively. Primers specific for the MCMV ie-1 gene and transcript differentially amplify DNA (476 bp) and RNA (257 bp). Positive DNA controls included serial dilutions of pRE1, a plasmid containing the relevant region of MCMV DNA (10^4, 10^3, 10^2, and 10^1 copies), and positive RNA controls consisted of RNA extracted from tissue culture cells infected with MCMV. Primers specific for the murine APRT were used as internal sample controls for both DNA and RNA amplification. Further details are presented under Materials and Methods.

chosen to detect MCMV ie-1 DNA and RNA transcribed from the ie-1 gene have been previously described (Yuhasz *et al.,* 1994). These can be used to differentiate PCR products derived from DNA and RNA by product size with the PCR product originating from viral DNA being 476 bp, while the product of cDNA derived from the messenger RNA is 257 bp (Keil *et al.,* 1987). As can be seen in Fig. 1, both ie-1 DNA and ie-1 RNA were readily detected in the buffy coat of mice acutely infected with MCMV, but not those mock infected. The amounts of DNA and RNA were well above our lower limits of detection of 10^2 copies each as determined by PCR and RT-PCR analysis of plasmid DNA and *in vitro*-transcribed RNA, respectively (data not shown). As another RT-PCR control, a sample of RNA extracted from MCMV-infected MEF cells was included in the reverse transcription and subsequent RT-PCR amplification (Fig. 1). To establish efficiency of extraction and amplification, a pair of primers homologous to sequences in the mouse APRT gene (Dush *et al.,* 1985) was used to amplify relevant sequences. As with the ie-1 primers, APRT DNA- and RNA-derived PCR products could be differentiated by size (374 and 186 bp, respectively). While there was some variation in the APRT signals between and within individual groups, which would be expected since the number of cells recovered in individual samples varied (range 2.3 $\times$ 10^6 to 1.1 $\times$ 10^7 cells), the levels in mock-infected mice were not less than those in infected animals.

Analysis of buffy coat from latently infected mice for MCMV ie-1 DNA and RNA

Having established our ability to detect both viral DNA and viral RNA in blood samples from acutely infected mice, we looked for viral DNA and RNA in latently infected mice. Blood was collected from infected and mock-infected mice 21 days p.i., and the DNA and RNA were separately extracted from each individual sample as for the acutely infected samples and analyzed by PCR and RT-PCR. The results presented in Fig. 2 are representative of the buffy-coat samples from four independent experiments. As with the samples from acutely infected mice, MCMV ie-1 DNA was easily detected in samples from MCMV-infected mice 21 days p.i. and absent in samples from mock-infected mice. However, MCMV ie-1 RNA was not detected in any of the samples tested by RT-PCR, suggesting that the DNA was in a latent state. As described under Materials and Methods, negative RT-PCR samples were further confirmed to be negative by using double PCR, a technique which increased our level of sensitivity to less than 10 template copies per sample (data not shown). Finally, the DNA

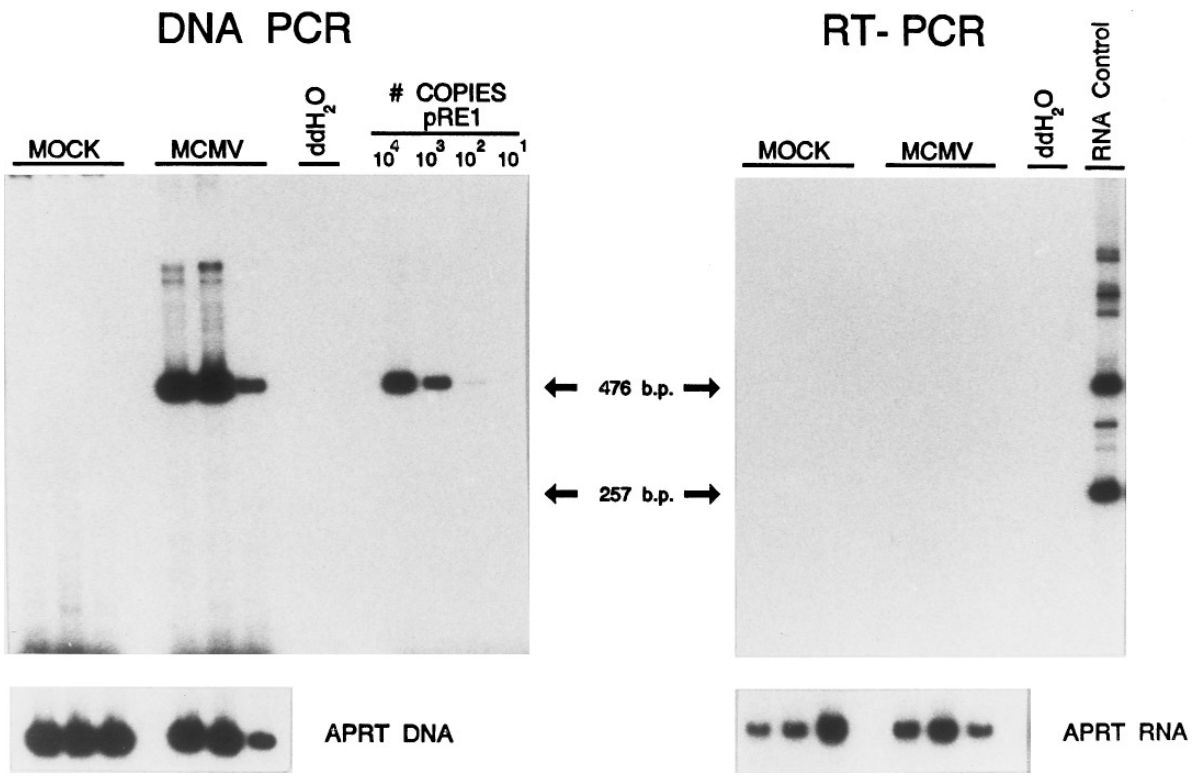

FIG. 2. MCMV ie-1 DNA and RNA in buffy coat of latently infected mice. Buffy-coat cells were collected from mock-infected mice and mice infected for 21 days with MCMV. The DNA and RNA were extracted and analyzed by PCR and RT-PCR, respectively, as described in the legend for Fig. 1.

PCR and the RT-PCR for mouse APRT indicated that there were no differences in extractions and amplifications.

Analysis of PBML and PMNL fractions from latently infected mice for MCMV ie-1 DNA and RNA

After determining that MCMV DNA persists in the buffy coat of mice, we began to define cell population(s) within the buffy coat which harbor the persisting MCMV genome. Initially, in four independent experiments, buffy coat cells of latently infected or mock-infected mice (three mice/group) were fractionated using Ficoll–Paque gradients into PBML and PMNL as matched sets. The DNA and RNA were extracted from each individual fraction and analyzed for MCMV ie-1 DNA and ie-1 RNA. As shown in Fig. 3A, the majority of the signal for the MCMV DNA in latently infected mice was detected in the PMNL fraction. However, positive signals for ie-1 DNA were also found in the PBML fraction although at reduced levels compared to the PMNL fraction. RT-PCR of all samples showed no ie-1 signal for RNA (Fig. 3B) and remained negative upon double PCR analysis (data not shown). APRT signals for both DNA PCR and RT-PCR were comparable for all samples.

To confirm that the signal in the PBML fraction was legitimate and not due to contaminating PMNL or other non-PBML cells ($\leqslant$6%), we used the "alternative" method described under Materials and Methods for recovery of PBML. This resulted in a ca. 5.1-fold increase in the number of cells and an increase in purity of PBML from 94% using the "original" fractionation to 99.6%. The DNA and RNA were extracted from PBML fractions processed from latently infected mice (21 days p.i.) in three independent experiments (three mice/group) and analyzed by PCR for MCMV ie-1 DNA and mouse APRT DNA. Figure 4 shows the results of the DNA PCR amplification of the PBML fractions isolated by the alternative method in comparison to PBML fractions isolated by the original method. Based upon densitometric scans of autoradiographs, the relative signal intensity for ie-1 DNA from the alternative method fractions increased 4.4-fold with an average 3.7-fold increase in APRT DNA signal.

Analysis of lymphocyte and monocyte fractions from latently infected mice for MCMV ie-1 DNA

The previous studies determined that PMNL and PBML fractions both contained cells positive for persistent MCMV DNA. The less intense signal in the PBML fraction indicated that only a small percentage or a subpopulation of cells in that fraction was responsible for harboring the DNA. We sought to further define the cell type(s) involved by additional fractionation of the PBML using FACS. PBML cells were obtained in three indepen-

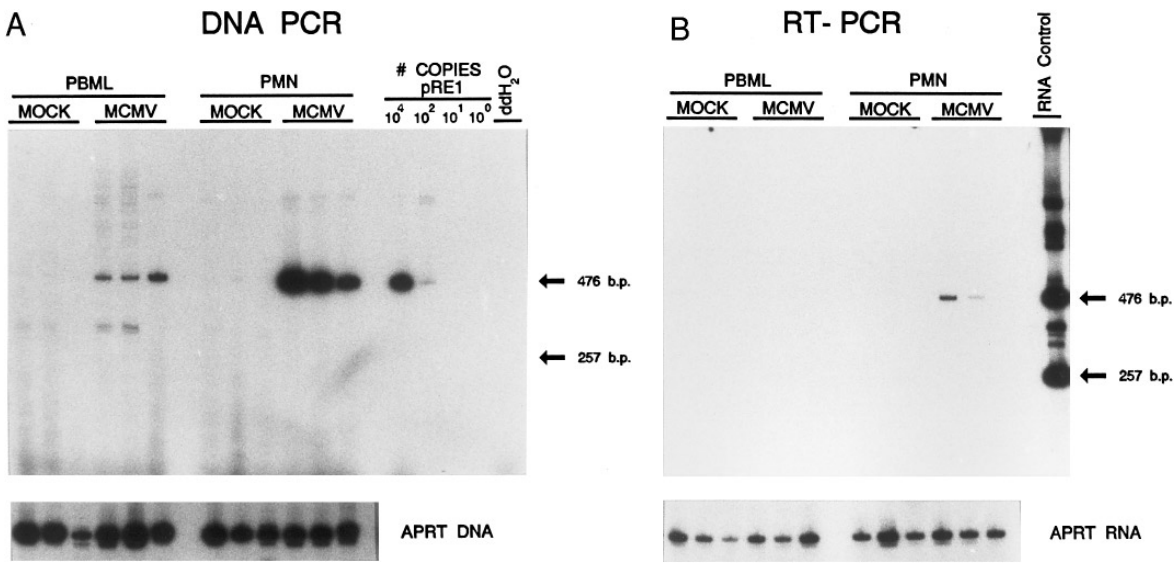

FIG. 3. MCMV ie-1 DNA and RNA in PBML and PMNL cell fractions of latently infected mice. Buffy-coat cells were collected from mock-infected mice and mice infected for 21 days with MCMV and separated into PBML and PMNL fractions using Ficoll–Paque gradients as described under Materials and Methods. The DNA and RNA were extracted and analyzed from matched fractions by (A) PCR and (B) RT-PCR, respectively, as described in the legend for Fig. 1. Positive DNA controls included pRE1, a plasmid containing the relevant region of MCMV DNA (10^4, 10^2, 10^1, and 10^0 copies).

dent experiments from latently infected or mock-infected mice (21 days p.i.) using the alternative method and three mice/sample were pooled (three samples/experiment). These samples were separated into monocytes and lymphocytes based upon staining for the CD11b surface antigen. As with other cell fractions, the DNA and RNA were extracted from the matched fractions and analyzed by double PCR and double RT-PCR. As Fig. 5 shows, no ie-1 DNA signal was detected in the Mac-1-negative (lymphocyte) fractions, but signal was obtained in the Mac-1-positive (monocyte) fractions. While standard PCR revealed slight signals for Mac-1-positive fractions (data not shown), double PCR for ie-1 DNA was utilized to amplify the signal obtained in the Mac-1-positive cells as well as to ensure a true negative signal in the Mac-1-negative cells. Negative results for double RT-PCR confirmed that DNA detected was not due to an active replicating infection and was consistent with a persistent/latent infection. These results indicate that monocytes were the cell type harboring the MCMV DNA in the PBML fraction.

DISCUSSION

We show here that at 21 days p.i. MCMV DNA persists in mouse buffy-coat cells and that both monocytes (Mac-1^+ PBML) and PMNL, but not lymphocytes (Mac-1^- PBML), harbor the MCMV DNA. Highly sensitive and specific PCR and RT-PCR using DNA/RNA size-differentiating primers were employed to analyze samples of buffy coat and subpopulations of blood cells from mice. When PBML were isolated by an alternative technique, the signal obtained in the PBML fraction was further confirmed and shown to not be due to PMNL or some other non-PBML cell such as endothelial cells contaminating this fraction. The possibility of contaminating endothelial cells was a concern since endothelial cells permissive to HCMV infection have been recently reported to be circulating in the blood of viremic humans (Grefte *et al.,* 1993; Percivalle *et al.,* 1993). As described under Materials and Methods, the alternative method resulted in approximately a 5.1-fold increase in the number of cells and an increase in purity of PBML from the original 94% to >99%. While this would result in a predicted 5.1-fold increase of APRT signal regardless of the presence of latent CMV, the ie-1 signal should increase ca. 5.4-fold if it were from latently infected PBML or decrease ca. 3.4-fold if the signal were from contaminating cells. As shown in Fig. 4, the ie-1 DNA signal increased with the alternative isolation method (4.4-fold with a 3.7-fold increase in the APRT signal). The increase, although slightly less than predicted, indicates the persisting CMV DNA was harbored in the PBML.

Our findings of MCMV DNA persisting in both PMNL and monocytes is in relative agreement with the findings of Collins *et al.* (1994) for acute infection. Although their findings were for stronger association with the monocytes than with the PMNL, these investigators found virus and viral DNA associated acutely with both cell types.

In the present study the relative number of copies of MCMV DNA was one for every 1×10^2 to 6×10^2 cells for both PBML and monocyte fractions. In contrast, the

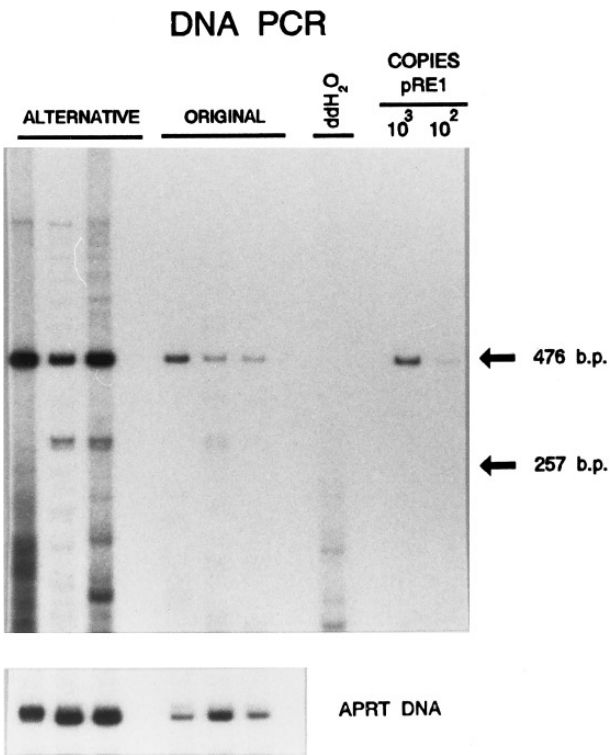

FIG. 4. Comparison of MCMV ie-1 DNA in PBML fractions of latently infected mice collected using the original and alternative methods of isolation. Buffy-coat cells were collected from mock-infected mice and mice infected for 21 days with MCMV and separated into PBML fractions by either the standard or the alternative method using Ficoll–Paque gradients as described under Materials and Methods. The DNA was extracted and analyzed by PCR as described in the legend for Fig. 1. Positive DNA controls included pRE1, a plasmid containing the relevant region of MCMV DNA (10^4 and 10^2 copies).

inability to detect MCMV DNA in the lymphocyte fraction using double PCR analysis shows that MCMV DNA must have been present at less than one copy per 1.7×10^4 cells, strongly indicating that this fraction was truly negative.

Before discussing the results further, it is important to consider three issues related to the CMV DNA detected. First, although viral ie-1 RNA could be detected by double PCR at a sensitivity of less than 10 copies of RNA per sample, it could be questioned whether the DNA detected in the samples represented DNA associated with replicating virus. As we detailed earlier (Yuhasz *et al.,* 1994), the associated ie-1 RNA in acute infections, in reactivated latent infections, and even in low-level persistent infections is well within measurable ranges when the amount of viral DNA detected in the present experiments is found. Acute infections and reactivated latent infections display RNA:DNA ratios greater than 1, while ratios for low-level persistent infections such as those in the lungs range from 0.018 to 0.24 (Yuhasz *et al.,* 1994). In the studies presented here, the RNA levels of all latently infected samples were below levels of detection indicating an RNA:DNA ratio below 1/10,000. This strongly suggests that cells in the samples tested were not productively infected.

A second concern is whether the DNA detected in the peripheral blood represented DNA that is potentially biologically active. This is one obvious requirement for latent DNA and it is typically tested by cocultivating potentially latently infected tissue or cells on permissive tissue culture cells in an attempt to recover reactivated virus. We have not been able to cocultivate the virus from peripheral blood (B. M. Mitchell and J. G. Stevens, unpublished results) and it is well established that HCMV cannot be recovered in this manner (Jordan, 1983). Although these results are compatible with the hypothesis that the DNA is not biologically active, it has been shown that freshly isolated monocytes are difficult to infect with HCMV and do not replicate virus (Einhorn and Ost, 1984; Rice *et al.,* 1984). This nonpermissiveness can, at least in part, be overcome since an increase in the number of cells permissive to HCMV infection results from extended culturing of peripheral blood monocytes with and without treatment of other agents (Ibanez *et al.,* 1991; Lathey and Spector, 1991; Soderberg *et al.,* 1993). These results, when combined with the knowledge that the incidence of transfusion-associated infection is reduced when blood

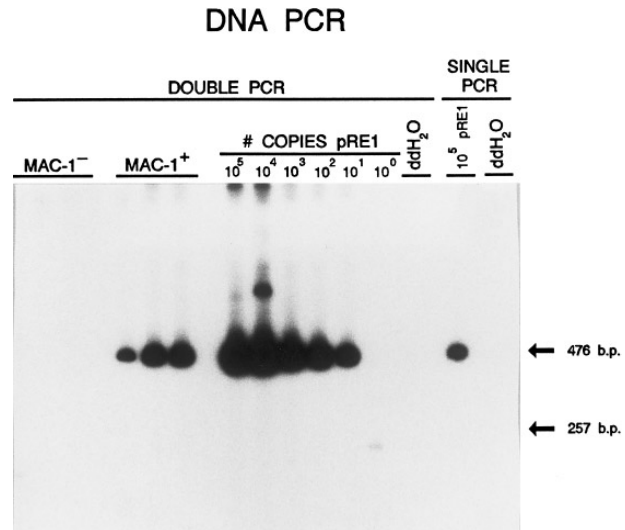

FIG. 5. MCMV ie-1 DNA and RNA in Mac-1^+ and Mac-1^- PBML fractions of latently infected mice. PBML cells were collected from the buffy coat of mice infected with MCMV 21 days p.i., using the alternate method. The PBML were combined as "three-mouse pools," stained with FITC-labeled Mac-1 monoclonal antibody, and separated by FACS into Mac-1^+ and Mac-1^- fractions. The DNA was extracted and analyzed from matched fractions by double PCR as detailed under Materials and Methods. Primers specific for the MCMV ie-1 gene amplify DNA as a 476-bp product. Positive DNA controls for the double PCR included serial dilutions of pRE1, a plasmid containing the relevant region of MCMV DNA (10^5 to 10^0 copies), while template-free ddH_2O served as a negative control. Included as positive and negative controls for the second PCR reaction in the double PCR analysis were 10^5 copies of pRE1 and ddH_2O, respectively (single PCR).

products are depleted of white blood cells (Yeager *et al.,* 1981), suggest that while peripheral blood cells may well be carriers of latent CMV DNA, additional differentiation and/or other signals may be necessary for the DNA to become active in reactivation. Transfusion-associated transfer of infection in the absence of detectable infectious virus represents additional evidence for a latent infection. The possibility of additional differentiation or signals being required is supported by the work of Taylor-Wiedemen *et al.* (1994) who showed that the induction of endogenous ie-1 and ie-2 HCMV gene expression in monocytes isolated from healthy CMV carriers can result from cellular differentiation *in vitro.* From these considerations it seems that the physiological state of involved cells is critical for virus replication and potential reactivation, and although we have not shown that the viral DNA detected is indeed "reactivatable," the proper conditions for facilitating this reactivation may not have been employed.

The final concern, and one that may be related to the second, is the possibility that since monocytes and PMNL both have phagocytic properties, the DNA detected had been phagocytosed by these cells. Although it cannot be ruled out, we do not favor this alternative, and for the following reasons. First, the discussion concerning the importance of physiological state of leukocytes which was just presented represents a reasonable explanation for viral restriction. Second, the relatively high levels of CMV DNA detected in the PMNL do not suggest phagocytosis. While lymphocytes recirculate between the blood, tissue, and lymph, PMNL do not return to the blood once they have migrated to other tissues. Because of this, if the MCMV DNA detected in these studies resulted from phagocytosis, the phagocytosis must have occurred in the circulatory system. Tests of mouse blood plasma 21 days p.i. for infectious virus using a standard MEF monolayer plaque assay (Yuhasz *et al.,* 1994) (sensitivity 1 PFU/0.8 ml of plasma adsorbed) and for viral DNA using PCR (sensitivity 10^2 copies/10 μl assayed) were negative for both (Mitchell and Stevens, unpublished results). Inability to detect virus or viral DNA in blood plasma does not suggest that the CMV DNA found was associated with scavenging within the circulatory system. Finally, this alternative seems more unlikely when it is remembered that PMNL and monocytes spend only a few hours to a few days in the circulation (Metcalf, 1989).

Our findings of MCMV DNA in monocytes and its absence in lymphocytes is consistent with results of Taylor-Wiedeman *et al.* (1991) for HCMV, but they differ from the findings involving PMNL (Taylor-Wiedman *et al.,* 1993). These results also contrast with those of Collins *et al.* (1993) who did not detect latent MCMV DNA in either whole blood or Ficoll–Hypaque-prepared blood fractions. While PMNL have been shown to harbor HCMV in viremic patients (Rinaldo *et al.,* 1977; Saltzman *et al.,* 1988; Dankner *et al.,* 1990; Gerna *et al.,* 1991; Revello *et al.,* 1992), Taylor-Wiedeman *et al.* (1993) found no HCMV DNA in PMNL samples taken from 10 healthy subjects (7 seropositive; 3 seronegative). These workers could detect 10 copies of HCMV DNA, a sensitivity which is at least 4 orders of magnitude greater than was required for us to detect the MCMV DNA in PMNL fractions. This difference between our findings and those of Taylor-Wiedeman *et al.* (1993) may be due to differences between time periods post-acute infection of our samples and theirs since there is evidence that, at least for MCMV, the number of latent-positive leukocytes decreases with time (Balthesen *et al.,* 1993) and this may be related to a differential loss in PMNL and monocytic populations. Additionally, as discussed below, it is possible that certain cells in the granulocytic lineage of mice, but not humans, continue to harbor latent virus.

The differences between our findings and those of Collins *et al.* (1993) are surprising; they were not able to detect latent MCMV DNA extracted from pooled whole-blood samples or from pooled PBML or PMNL of mice 104 days after infection. While their analysis was performed at a later time point than ours, others have been able to detect MCMV DNA in blood leukocytes as late as 12 months postinfection (Balthesen *et al.,* 1993). It may be important to point out that Belthesen *et al.* (1993) inoculated neonates since the age of the animal may influence the dynamics of the infection as supported by other studies (Collins *et al.,* 1993). However, the age of the mice used in the studies presented here were similar to the age used by Collins *et al.* (1993). One possible explanation for the dissimilarity could be the route of infection. Collins *et al.* (1993) infected the mice by intraperitoneal injection, which requires replication in the peritoneum before spread to other tissues occurs, and we infected by tail vein injection. The latter route may be more efficient in seeding multiple organ systems, including bone marrow. Since blood leukocytes are only transiently present in the circulation (Metcalf, 1989), CMV DNA must be continuously "seeded" into them if a long-term persistence is to occur. Bone marrow is a likely candidate as the source for these latently infected cells, and this was not tested by Collins *et al.*

It seems quite possible then, that progenitor bone marrow cells are latently infected, maintain the viral DNA, and subsequently replicate and transmit the DNA as they divide and terminally differentiate. Several observations made by others support this concept. First, Kondo *et al.* (1994) showed that latent HCMV can be detected in myeloid bone marrow cells ($CD14^+$, $CD15^+$, $CD33^+$) that serve as progenitors for both peripheral blood granulocytes and monocytes. It is also known that HCMV infects $CD34^+$ bone marrow cells (Minton *et al.,* 1994) which are precursors for both granulocytes and monocytes. Finally, CD13, a cellular surface molecule on granulocytes,

monocytes, and their bone marrow precursors, has been reported to mediate HCMV infection (Soderberg *et al.,* 1993). Currently, we are separating and analyzing the bone marrow cells of mice latently infected with MCMV to determine whether myeloid stem cells do in fact harbor MCMV DNA. Whatever these experiments demonstrate, it is clear from the present study that in latently infected mice, MCMV DNA in peripheral blood is detectable only in monocytes and PMNL.

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