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Lungs Are a Major Organ Site of Cytomegalovirus Latency and Recurrence

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Recurrence of infectious virus from the latent viral genomes is the initiating event in the pathogenesis of cytomegalovirus (CMV) disease during states of immunodeficiency. Interstitial pneumonia is a frequent manifestation of posttransplantation CMV disease, in particular after bone marrow transplantation and heart and lung transplantations. Recurrence can occur within the transplant derived from a latently infected donor as well as within latently infected organs of the transplant recipient. The reason for a predilection of the lungs as a site of CMV pathology is so far unknown. In a murine model of CMV latency, the lungs were identified as an authentic site of latent infection, since the viral genome remained detectable in lung tissue even after it was cleared to an undetectable level in blood and bone marrow. A comparison between the lungs and the spleen, the previously most thoroughly investigated site of murine CMV latency, revealed a 10-fold-higher burden of latent viral genome for the lungs. Most important, the organ-specific risk of in vivo recurrence was found to correlate with the organ-specific viral genomic load. This new finding thus characterizes the lungs as a high-risk organ for CMV recurrence, and this fact may explain in part why interstitial pneumonia is a frequent manifestation of recurrent CMV infection.

Cytomegaloviruses (CMV) are members of the betaherpesviruses (29). It is a common feature of herpesviruses that acute infection is not terminated by clearance of infectious virus and the viral genome but is followed by a phase of persistent virus replication that can vary largely in duration and is often confined to a particular tissue site. Eventually, virus is retained in a latent state defined operationally by the inability to detect infectious particles and by the possibility of inducing a recurrent infection (30).

Human CMV is highly prevalent in the population. It does not cause overt disease in immunocompetent hosts, but immunocompromised patients are at risk of severe and often fatal manifestations of CMV disease (38). Specifically, primary infection during pregnancy is responsible for birth defects (3), CMV contributes to the syndrome in the terminal stages of AIDS (11), and CMV recurrence is the major viral complication in bone marrow transplantation (9, 23) as well as in organ transplantation, including liver, kidney, and heart and lung transplantations (12). It is established that recurrence within the transplantation recipient as well as from the transplant both contribute to the overall risk of CMV disease. Collectively, the clinical data indicate that a variety of organs, and in particular also cells in blood and bone marrow (28, 34, 37), can serve as a source of latent CMV.

Murine models of CMV latency have been employed to address specific questions experimentally (reviewed in reference 16). The interest was focused on the spleen as a site of murine CMV latency, and recent work has pointed to the sinusoidal lining cell of the splenic stroma as a candidate for the cell type that may harbor the latent genome (21, 26). However, studies of transmission by transplantation (10, 31), recurrence in splenectomized mice (16), in vitro recurrence (7, 39), and amplification of the viral genome (18) have suggested various other organs as additional sources for latent virus. Collectively, data in the model revealed a good fit to human CMV with respect to organ sources of latent virus. This finding has led to the belief that multiple organs are true sites of CMV latency. However, the apparent possibility that contamination by latently infected circulating or temporarily tissue-residing blood-borne cells may account for the multiple organ transmission was never controlled for rigorously. To date, the lungs have not been taken into consideration as an organ site of CMV latency, even though interstitial pneumonia is a significant manifestation of CMV disease both in patients (23) and in murine models (15, 27, 35).

In this report, the lungs are identified as an authentic site of murine CMV latency by the detection of a high-copy density of viral DNA in lung tissue at a stage of latent infection at which the viral genome in blood had fallen below the detection level. The incidence of experimentally induced in vivo recurrence in the lungs and spleen correlated with the tissue burden of the latent viral genome, which was found to be 10-fold higher in the lungs. The high risk of recurrence in the lungs offers an explanation for the high incidence of interstitial pneumonia among the diverse manifestations of CMV disease.

MATERIALS AND METHODS

Virus and infection conditions. Experiments were performed with the original Smith strain (36) of mouse CMV distributed by the American Type Culture Collection under the product number ATCC VR-194, for which the physical map was determined by Ebeling et al. (8). The virus was propagated in mouse embryo fibroblast culture and partially purified by sedimentation through a 15% sucrose cushion to preclude the unspecific toxic effects that are associated with virus preparations obtained from salivary glands (6). BALB/c mice, bred and housed at our facility under speci-

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fied pathogen-free conditions, were infected as indicated in "Protocol for establishing latent infection" by using a stock of purified virus that had a titer of 1.5×10^9 PFU/ml in 15% sucrose solution in Tris-HCl buffer (pH 7.8) containing 20 mM Tris, 83 mM KCl, and 2 mM EDTA. For in vivo application, dilutions were made in isotonic saline immediately before use.

Induction of virus recurrence. Recurrent infection was induced by total-body gamma irradiation of latently infected mice with a single dose of 6 Gy from a cesium-137 source delivering a dose rate of 0.708 Gy min^{-1}.

Processing of organs and blood samples for DNA isolation. Organs were taken under aseptic conditions, minced, and transferred into 1.5 ml of digestion buffer composed of 10 mM Tris HCl (pH 8.0) containing 100 mM NaCl, 25 mM EDTA, 0.5% (wt/vol) sodium dodecyl sulfate (SDS), and 1 mg of proteinase K (Boehringer GmbH, Mannheim, Germany) ml^{-1} added immediately before use. Samples were incubated for 12 to 16 h at 37°C in a shaking water bath. After the addition of 0.5 mg of fresh proteinase K per ml, digestion was continued at 56°C for 2 h. Clumps were resolved by douncing. Bone marrow cell suspensions were obtained by flushing phosphate-buffered saline (PBS) through the shafts of femora and tibiae. The cells were washed three times with PBS by sedimentation ($200 \times g$) and resuspension before transfer into digestion buffer. The digest was performed at 56°C for 2 h. Blood was taken by heart puncture and transferred into PBS (devoid of $MgCl_2$ and $CaCl_2$) containing 0.1% (vol/vol) EDTA to prevent coagulation. Processing for whole blood DNA was done as with bone marrow. For titration of blood leukocytes, cells were washed three times and erythrocytes were removed by hypertonic lysis. The leukocytes were not subjected to a density gradient in order not to lose a relevant subpopulation. Cytofluorometric sorting followed by polymerase chain reaction (PCR) did not reveal viral DNA in the fraction of residual erythrocytes or platelets. Viral DNA was present in mononuclear leukocytes as well as in $Gr\text{-}1^+$ $CD11b^+$ polymorphonuclear leukocytes (data not shown). Graded numbers of leukocytes were digested separately in 0.3 ml of digestion buffer-proteinase K. Digests from low cell numbers ($\leq 10^5$) were supplemented with 3 μg of certified CMV-negative carrier DNA taken usually from the spleen. DNA was isolated by standard procedures, including phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. The amount of purified DNA was determined from the optical density at 260 nm.

Amplification of viral genomic sequences. For detection of the viral genome in tissues of latently infected mice, specific DNA sequences were amplified by PCR on the basis of published methods (32), with some modification. Contamination was avoided by following accepted recommendations (19).

(i) Oligonucleotide primers and probes. As a representative of viral DNA, a 363-nucleotide sequence was amplified from exon 4 of the murine CMV immediate-early gene *ie1*. The *Hin*dIII map of the viral genome (8), the location as well as the exon/intron organization of the *ie1* gene therein (17), and the positions of the chosen oligonucleotides are outlined in the top, center, and bottom, respectively, of Fig. 1. Viral oligonucleotides are designated by the gene's name, followed by the initial 5′ nucleotide with the counting starting at the 5′ cap site of the respective mRNA. Primers were as follows: forward primer IE1.1983, 5′-ATTGTTCATTGCCT GGGGAGTTT-3′; reverse primer IE1.2345, 5′-ATCTGGTG CTCCTCAGATCAGCTAA-3′; probe IE1.2135, 5′-AGCG TATCCACACATGTGGTAGT-3′. A 419-nucleotide sequence of the murine CMV) early gene *e1* (5) was amplified by using oligonucleotide E1.1143 from exon 2 as the forward primer and oligonucleotide E1.1561 from exon 3 as the reverse primer. Oligonucleotide E1.1353 from intron 2 served as the hybridization probe. As a cellular gene control, oligonucleotide sequences were chosen from the cDNA sequence of the mouse β-actin gene (1). Oligonucleotides 5′-AGCCATGTACGTAGCCATCCAGGCT-3′ and 5′-GGA TGTCAACGTCACACTTCATGATGG-3′ served as the forward and reverse primers, respectively. Oligonucleotide 5′-GCTCTAGACTTCGAGCAGGAGATGGCCACT-3′ was used as the probe. All oligonucleotides were synthesized on an automated DNA synthesizer (model 381A; Applied Biosystems, Weiterstadt, Germany) and purified on Sephadex G25 columns (Pharmacia, Freiburg, Germany). Viral primers were pretested to give no amplification product of critical size from mouse genomic sequences by using organ DNA from uninfected mice as the template.

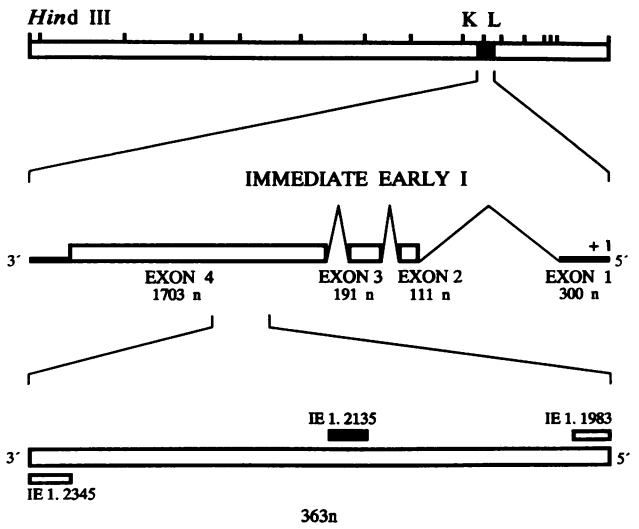

FIG. 1. Map position of the PCR-amplified sequence. Shown are the *Hin*dIII cleavage map of the murine CMV (Smith strain) genome (top), the structure of immediate-early gene *ie1* (center), and the positions of the PCR primers and the probe (bottom). The lengths of sequences are given in nucleotides (n).

(ii) DNA amplification. In general, samples of 3 μg each of DNA were subjected to PCR in a total reaction mixture volume of 0.1 ml. For high numbers of blood leukocytes, a respective aliquot of the isolated DNA was tested, whereas for low numbers, the DNA was supplemented with carrier DNA. The amplification buffer was 67 mM Tris HCl (pH 8.8) containing 10% (vol/vol) dimethyl sulfoxide, 0.016% (wt/vol) bovine serum albumin, 3 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 7 mM 2-mercaptoethanol, and 0.2 mM each of the four deoxyribonucleoside triphosphates. The two primers were added at final concentrations of 0.2 μM each. After heating to 95°C for 5 min, 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was added. The PCR was performed in an automated thermal cycler (Thermocycler model 60; Bio-Med, Theres, Germany). Amplification cycles entailed denaturation at 94°C for 1.5 min, annealing of primers at 55°C for 2.5 min, and primer extension at 72°C for 2 min that was extended to 6 min in the 35th cycle.

(iii) Analysis of PCR-amplified DNA. Hybridization with probe IE1.2135 was used to verify the identity of the amplification product. Samples of 10 μl each (1/10 of the

reaction volume) were electrophoresed on 1.4% agarose minigels at 50 V for 1.5 h. Gels were washed twice for 15 min each with transfer solution (1 M NaCl in 0.5 N NaOH) before 12 to 16 h of Southern transfer onto GeneScreen Plus nylon filters (Du Pont NEN, Bad Homburg, Germany). The filters were washed twice for 1 min each in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and baked for 60 min at 80°C. After a further washing with 0.1× SSC–1% (wt/vol) SDS for 60 min at 65°C, prehybridization was carried out for 1 h at 58°C in hybridization buffer composed of 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4]), 7.5× Denhardt's solution, 0.1% (wt/vol) SDS, and 10 μg of salmon sperm DNA ml^{-1}. Hybridization was carried out for 12 to 14 h at 58°C with the respective ^{32}P-end-labeled oligonucleotide probes. The filters were washed with 0.2 and 0.1 M sodium phosphate buffer (pH 7.2)–1% (wt/vol) SDS for 30 min at 58°C and for 15 min at 56°C, respectively. Autoradiography was done, depending on the signal strength, for up to 24 h at −70°C with two intensifying screens (Cronex; Du Pont) and Kodak X-AR film.

Sensitivity and semiquantitative evaluation of the PCR. Samples with all ingredients except template DNA served as the negative control. To determine the sensitivity of the PCR, defined copy numbers of plasmid pIE111 were mixed with 3 μg of certified negative chromosomal mouse DNA from organs of uninfected mice. The circular plasmid pIE111 encompasses genes *ie1* and *ie3* of murine CMV (22). Linearization of the plasmid did not change the sensitivity of detection. The PCR products were quantitated by densitometric scanning and computing from the autoradiographs with a Joyce-Loebl scanner. It was not intended in our experiments to determine precise copy numbers of viral DNA in host tissues but rather to rank test signals as <10, 10, 100, or >100 copies.

RESULTS

Protocol for establishing latent infection. Human CMV is acquired mainly perinatally and during early childhood. Accordingly, previous murine models of CMV latency were based on experimental infection of neonates or of weanling mice (reviewed in reference 16). We used here the model of neonatal infection. BALB/c mice were infected intraperitoneally with 10^2 PFU of purified mouse CMV at the first day after birth; the mice were referred to as latency-type N mice. BALB/c is a mouse strain that is genetically susceptible to infection with mouse CMV (33), and, according to our experience, the Smith strain is highly virulent in BALB/c newborns even when purified from cell culture. Thus, mortality was 40% on average, with death occurring typically in the third week. Survivors showed runting until 4 to 6 weeks after infection, recovered, and appeared phenotypically normal later on. By the time of death, virus had replicated in a variety of vital organs, whereas, in the survivors, virus replication was confined to the salivary glands at 1 month after infection. Restricted to that specific site, persistent infection continued for about 4 months before it declined, and, beyond 6 months postinfection, virus was no longer detectable in homogenates of salivary glands, lungs, and spleen. Specifically, no virus was detected at 1 year postinfection in any of these organs in a total of 40 neonatally infected mice tested (Table 1) when the most sensitive technique was employed, namely, the in vitro plaque assay with centrifugal enhancement of infectivity (13). To fully exploit the sensitivity of this assay, 100% of the organ homogenate was plated on permissive mouse embryo fibroblast monolayers with no semisolid overlay. The prevalence of low-level persistent infection was therefore <2.5%. Only in rare cases was virus detected by this method at times later than 6 months, and it remained open to question whether these represented individual instances of prolonged virus persistence or episodes of spontaneous recurrence. In conclusion, beyond 6 months postinoculation, cases of low-level persistent infection are negligible.

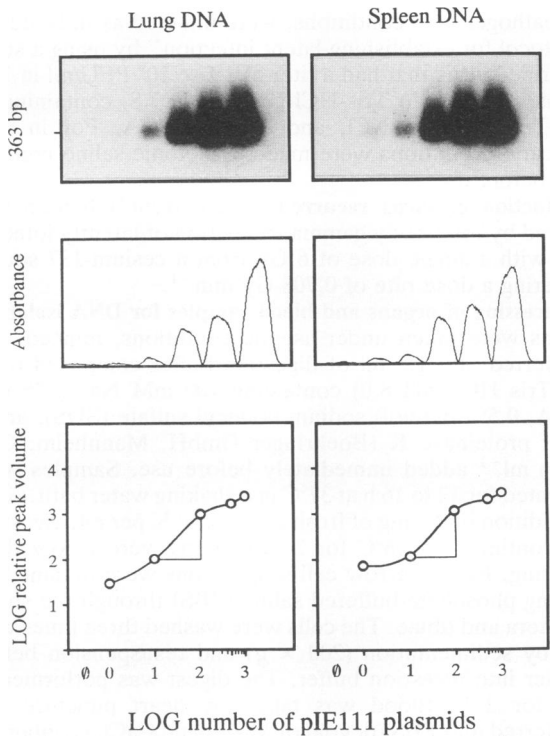

FIG. 2. Determination of PCR assay sensitivity and linearity. Graded numbers of plasmid pIE111, which includes the 363-nucleotide test sequence from exon 4 of gene *ie1*, were mixed with 3 μg of carrier DNA derived from normal lungs (left panel) or spleen (right panel). Titration was started with 1,000 copies (13.4 fg) of pIE111. (Top) Autoradiography of the amplified DNA with the ^{32}P-end-labeled probe IE1.2135. (Center) Densitometric scanning of the corresponding autoradiographs. (Bottom) Log-log plots of signal intensity as a function of pIE111 copy number. ∅, Negative control with carrier DNA but no plasmid.

Sensitivity of detection of a viral test sequence within organ DNA. The viral immediate early gene *ie1* (17) was chosen as a marker gene for testing the presence of viral DNA in organs of latently infected mice. To determine the sensitivity of the detection of *ie1* by PCR (recall Fig. 1) within cellular DNA of different organ sources, graded numbers of the *ie1*-containing plasmid pIE111 were titrated to a constant amount of cellular DNA from normal lungs or spleen and a 363-bp sequence from exon 4 of gene *ie1* was amplified in 35 cycles (Fig. 2). The endpoint titration revealed a clearly visible autoradiographic signal with 10 copies of the plasmid and a faint signal with a single copy (Fig. 2, top). The sensitivity was independent of the organ source of the carrier DNA, lung DNA or spleen DNA (Fig. 2, left versus right). Essentially the same result was obtained with blood leukocyte DNA and bone marrow cell DNA as the carrier DNA (data not shown).

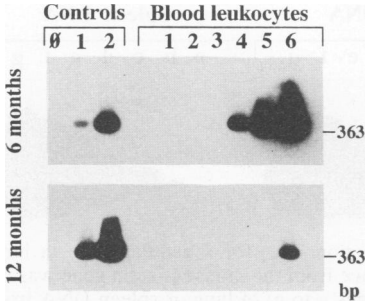

FIG. 3. Frequency of blood leukocytes carrying the CMV test sequence. Blood leukocytes from pools of blood from five mice were tested for the presence of CMV DNA by PCR at 6 months (top) and 12 months (bottom) after neonatal infection. The cells were titrated before the DNA was isolated. ∅, All reagents except viral templates. Control lanes 1 and 2, 10 and 100 molecules, respectively, of pIE111 as viral control templates mixed with 3 μg of spleen carrier DNA. Test lanes 1 to 6, DNA from 10^1 to 10^6 leukocytes, respectively, supplemented with carrier DNA for cell numbers $\leq 10^5$.

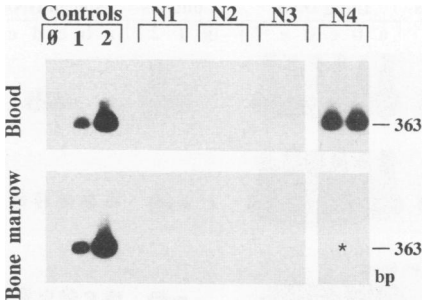

FIG. 4. Test for viral DNA in blood and bone marrow. The viral test sequence was amplified by PCR from whole-blood and bone marrow cell DNA prepared from neonatally infected mice at 12 months of age. Mice N1 through N4 were tested individually in duplicate with 3-μg samples of DNA. ∅, All reagents except DNA. Control lanes 1 and 2, 10 and 100 copies, respectively, of plasmid pIE111 mixed with 3 μg of the respective N1 DNA. *, sample not tested.

Densitometric scanning of the autoradiographs (Fig. 2, center) and computing of the signal volumes revealed a nonlinear relationship between plasmid copy numbers and the amount of PCR product in a log-log plot (Fig. 2, bottom). The relationship is linear only between 10 and 100 copies, as is indicated by the finding that 1 log change on the abscissa results in 1 log change on the ordinate. Therefore, 10 and 100 copies of pIE111 were routinely used as positive controls in the subsequent experiments.

Delayed clearance of the viral genome from the blood. There is preliminary evidence that multiple organs harbor latent CMV (reviewed in references 16 and 24). However, one must envisage the possibility that the latently infected cell is not intrinsic to a particular tissue but is just a passenger. This objection is particularly relevant, since blood-borne cells are a known source of latent CMV (2, 25). While perfusion of organs may more or less quantitatively remove cells migrating in blood vessels (10), blood-borne cells temporarily residing in tissues are difficult to exclude formally, except if organs are positive for the viral genome at a stage of latency at which the viral DNA is cleared from the blood.

We have therefore tracked the presence of viral DNA in blood leukocytes at different times after termination of viral replication in organs by PCR amplification of the 363-bp test sequence from gene *ie1* (Fig. 3). At each time point, blood leukocytes were pooled from five mice scored individually to have cleared infectious virus from salivary glands, lungs, and spleen. The frequency of leukocytes carrying viral DNA was estimated by endpoint titration of the cells. At 6 months postinfection, that is, shortly after termination of virus persistence in the salivary glands, ca. 10 to 100 copies of the viral sequence were detected in the DNA from 10^4 leukocytes, whereas no signal was obtained from 10^3 leukocytes (Fig. 3, top). The minimum estimate for the frequency of positive cells is therefore 10^{-4}. At a later stage, at 1 year postinfection, corresponding to 6 months after cessation of virus replication, the viral genome was still detectable, although in a 100-fold-lower frequency of leukocytes (Fig. 3, bottom). IE1 protein antigenemia was not visible at either stage (data not shown).

Because, in a pool of mice, detection of the viral test sequence can result from a mixture of positive and negative samples, individuals were also tested separately at 1 year postinfection (Fig. 4). In this particular experiment, the plasmid controls were made with blood leukocyte DNA and bone marrow cell DNA derived from mouse N1 as the carrier DNA to ensure that amplification was not inhibited by factors in the DNA preparation. In three of four animals (Fig. 4, N1 to N3), no viral DNA was detected in two independent 3-μg samples of leukocyte or bone marrow cell DNA, while individual N4, even though it was also negative for infectious virus in organs, was positive as indicated by both blood samples, with ca. 100 copies per sample (Fig. 4, N4). Mouse N4 was therefore excluded from the analysis of latent viral DNA in organs. It should be noted that the two samples represented 50% of the yield of DNA from the blood of mice at 1 year of age. From this result and the assay sensitivity, it can be calculated that the total copy number in the blood of mice N1 to N3 was <20 copies. The virtual absence of viral DNA in the blood provided additional evidence against a low-level persistent infection in individuals N1 to N3, as persistent infection should have resulted in continued delivery of viral DNA to the blood.

The lungs and spleen are authentic sites of murine CMV latency. Since a significant contamination of organs by latently infected blood leukocytes was excluded for mice N1 to N3 at 1 year postinfection (Fig. 4), these mice served to answer the principal question of intrinsic CMV latency in organs independent of blood-borne passengers. Previous investigators have noted that the latent CMV genome in the spleen is not detectable by in situ cytohybridization (21, 26) or by Southern blotting without prior amplification (18). Therefore, as described for the blood, amplification by PCR of the *ie1* gene test sequence was employed to detect viral DNA in organs. We focused on the comparison between the lungs and spleen. The lungs were chosen because of their relevance in viral pathology, and the spleen was chosen because it was considered a major site of murine CMV latency on the basis of in vitro virus recurrence from explant cultures (reviewed in reference 16). Both organs clearly harbored latent CMV DNA (Fig. 5). In the lungs, there was almost no variance among individuals and also no apparent sample variance. All three individuals tested showed a viral sequence copy number of >100 in each of six independently amplified 3-μg samples of tissue DNA (Fig. 5, left). In contrast, the copy number in the spleens of the same individuals was apparently lower, ca. 10 to 100 copies per

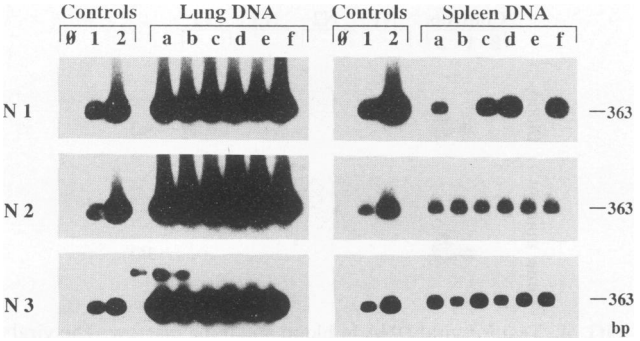

FIG. 5. Latent CMV load in lungs and spleen. Mice N1 to N3, certified to be negative for viral DNA in blood and bone marrow at 12 months after neonatal infection, were tested individually for the presence of the viral sequence in the lungs (left) and spleen (right). Lanes a to f, six independently amplified 3-μg samples of organ DNA.

sample, and in the case of mouse N1 not all samples were positive. It should be noted that the viral sequence was also detected in salivary glands and adrenal glands (>100 copies per sample), the heart (ca. 100 copies), and the kidneys (≤10 copies) (unpublished data). If one considers only the lungs, a minimum of 600 copies was thus contained already within the tested 18 μg of lung DNA, which can be extrapolated on the basis of the DNA yield to $>10^4$ copies in the whole organ. As the blood of the same individuals contained <20 copies in total, this experiment proved the existence of multiple authentic organ sites of murine CMV latency.

High load of the latent viral genome in the lungs. Because most previous studies have focused on the spleen as the major site of murine CMV latency, the apparently higher prevalence of viral DNA in the lungs came as a surprise and needed to be quantitated and controlled for more thoroughly. Endpoint titration starting with 3 μg of tissue DNA from mouse N2 revealed 30 ng of lung DNA as the detection limit for the viral test sequence, whereas the detection limit was 300 ng with spleen DNA (Fig. 6). Furthermore, this 10-fold difference was corroborated by the comparison of the signal intensities at any step in the titration.

It is critical to exclude the possibility that the apparent signal difference just reflects a technical difference in the amplification efficacies of lung and spleen DNA. As the physical state of the viral genome during latency is unknown, it is open to question whether it is circular, a state found as a replicative intermediate in productive infection (20), or whether it is integrated into the cellular genome. Controls should account for both possibilities. That a circular plasmid containing the test sequence, namely, plasmid pIE111, is amplified with the same efficacy from lung and spleen DNA has been documented above (recall Fig. 2). To account for the second possibility, a cellular gene sequence, a sequence from mouse β-actin (1), was amplified from the same DNA preparations used for Fig. 6 for the detection of the CMV sequence (Fig. 7). With a detection limit of 30 pg, the amplification efficacy for the cellular test gene was the same for lung and spleen DNA. This excluded an influence of the organ DNA source on the technical amplification efficacy. In conclusion, the lungs harbored approximately 10-fold more latent viral DNA relative to cellular DNA than did the spleen.

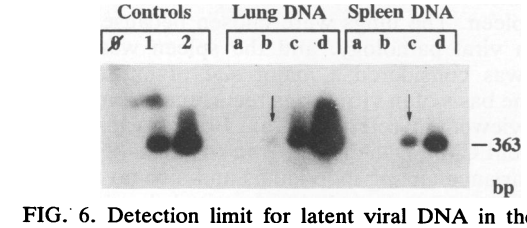

FIG. 6. Detection limit for latent viral DNA in the lungs and spleen. The viral test sequence was amplified from 3 ng to 3 μg (lanes a to d) of lung or spleen DNA from mouse N2. The total amount of DNA was adjusted by negative spleen carrier DNA to 3 μg. The detection limits are marked by arrows.

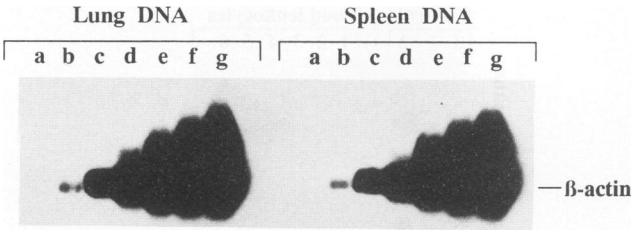

FIG. 7. Detection limit for a cellular gene in lung and spleen DNA. A sequence from the mouse β-actin gene was amplified from 3 pg to 3 μg (lanes a to g) of lung or spleen DNA from mouse N2.

The lungs represent a major site of in vivo CMV recurrence. The detection of a viral test sequence in tissue does not indicate whether the genome is complete and functional for reactivation and virus recurrence. The speculation that the regulatory gene *ie1* may have been selectively retained in cells was refuted by amplification of another viral sequence belonging to gene *e1* (data not shown). However, critical deletions in the genome are hereby not excluded, and testing more genes by PCR would add little to solve this principal problem. We have therefore determined organ-specific incidences of recurrence in vivo after general immunosuppression and ablation of hematopoiesis by total-body gamma irradiation (Table 1). This suppression regimen is related to the hematoablative conditioning in clinical bone marrow transplantation, after which human CMV recurrence arises as a major problem. In two experiments with latently infected mice at 1 year after neonatal infection, no virus was detectable in lungs or spleen (or salivary glands; data not shown) when no treatment was given. The prevalence of prolonged persistence or the incidence of spontaneous recurrence was therefore <5% (0 of 20 mice) in each experiment. By contrast, taking both experiments together, the incidence of induced recurrence was 12.5% (5 of 40 mice) for

TABLE 1. Risks of murine CMV recurrence in lungs and spleen

Group[a]	Expt no.	Incidence of recurrence in:		Range of virus titer (log PFU) in:	
		Lungs	Spleen	Lungs	Spleen
Latent	1	0/20 (<5%)	0/20 (<5%)		
	2	0/20 (<5%)	0/20 (<5%)		
Recurrent	1	8/20 (40%)	3/20 (15%)	2.7–3.3	2.1–2.9
	2	6/20 (30%)	2/20 (10%)	2.5–3.2	2.2–2.7

[a] Groups of 20 neonatally infected BALB/c mice at 1 year of age were either left untreated (latent group) or were gamma irradiated with a dose of 6 Gy (recurrent group). Virus titers per organ were determined 2 weeks later from organ homogenates by plaque assay, with centrifugal enhancement of infectivity.

the spleen and 35% (14 of 40 mice) for the lungs. It should be noted that virus titers in recurrently infected organs were in the order of 1,000 PFU, while the negative organs did not show a single plaque when all of the material was plated. The scoring was therefore not obscured by assay sensitivity. Rather, recurrence appeared to be a stochastic event that occurred in the lungs with a higher frequency than in the spleen.

In conclusion, the organ-specific risk of in vivo recurrence reflects the organ-specific latent virus burden, and, according to both criteria, the lungs are a significant site of murine CMV latency.

DISCUSSION

We have combined the detection of latent viral DNA by PCR with the induction of in vivo recurrence to identify organ sites of CMV latency in the murine model. Four main findings are reported. (i) Viral DNA is maintained in blood leukocytes for up to 6 months after the termination of productive infection in tissues but is eventually cleared as indicated by a progressing decline in the frequency of latently infected leukocytes. (ii) At an advanced stage of latency, at which viral DNA in the blood has fallen below the limit of detection, it is still prevalent in organs. (iii) The burden of organs with latent CMV, that is, the average viral copy number in tissue, correlates with the organ-specific incidence of recurrence. (iv) The lungs represent a high-risk organ for CMV latency and recurrence.

That leukocytes are a notable source of latent CMV was proposed long ago for both murine (2, 25) and human (28, 34) CMV, and this idea is also supported by clinical experience of human CMV transmission by blood transfusion. Our data show that viral DNA is maintained in blood leukocytes for an extended period after resolution of virus replication in tissues but that the number of positive leukocytes decreases with time. Apparently, leukocytes represent only a temporary site of CMV latency. This idea is reasonable given the fact that leukocytes are continuously renewed by hematopoiesis. If newly generated cells that replace latently infected cells do not get infected during episodes of recurrence, the frequency of latently infected cells must necessarily decline. That this decline is indeed observed suggests that spontaneous recurrence is not a significant event in latently infected mice.

Solid organs harbor leukocytes not only in the vascular compartment, from which they can be removed by perfusion, but also in the interstitial compartment. This is true in particular for monocytes and histiocytes that are capable of entering tissues by diapedesis. The detection of CMV DNA in organs by PCR (18) as well as the transmission of CMV by clinical (12) or experimental (10, 31) organ transplantation can therefore not be taken per se as evidence for multiple authentic organ sites of CMV latency. The possible contribution of leukocytes needs to be evaluated carefully before conclusions on organ sites of CMV latency can be drawn.

In murine models of CMV latency in the spleen, approaches to localize the latent genome by cell separation followed by in vitro coculture reactivation (21) or by PCR (26) have not yet led to an unequivocal conclusion. A sinusoidal lining cell of the splenic stroma with an unusual phenotype, for which a counterpart has also been described for the human spleen stroma (4), was identified in situ as a productively infected cell type during acute infection (21), and the stroma was found to be enriched with the latent genome (21, 26). Yet, in the authors' own interpretation, the stromal fraction harboring latent CMV was still a composition of a variety of cell types, including blood-borne cells, which all stay in question as candidates for the cellular site of CMV latency in the spleen. As the spleen is a large reservoir of leukocytes, studies of a likely CMV latency in the splenic stroma may be obscured by contamination with latently infected circulating and homing leukocytes. Our finding that viral DNA remained detectable in the spleen after its clearance in blood can be taken as additional supportive evidence for the existence of true virus latency in the spleen stroma.

While, because of the ease of inducing recurrence in vitro, the spleen was the most intensively studied organ site of latency in the murine model, other organs would be more appealing in regard to human CMV pathogenesis during recurrence in vivo. We consider it an important aspect that after clearance of the viral genome from the blood, the lungs harbored 10-fold more viral DNA than did the spleens of the same individuals. It is instructive to give an idea about the frequency of latently infected cells in the lungs. A sample of 3 μg of organ DNA represents the DNA content of ca. 5×10^5 diploid tissue cells. Thus, ca. 100 copies of viral DNA per sample give a maximum estimate of one latently infected cell among 5,000 tissue cells, that is, a frequency of 200×10^{-6}. It is self-evident that the frequency is even lower if the copy number per cell is >1. From this calculation it can be predicted that a histological in situ analysis of CMV latency will be difficult to achieve.

While blood-borne circulating or temporarily homing cells were excluded as cellular sites of virus latency by our approach, long-term-resident, aged histiocytes must remain in the discussion. It may be speculated that differences in the homing patterns of histiocytes may account for the observed organ differences in the prevalence of latent viral DNA and risk of recurrence. However, recent work has provided evidence for macrophages serving as a source of CMV amplification and dissemination rather than being the cellular site of CMV latency and recurrence (14). If the latently infected cell in the lungs is a sinusoidal lining cell or endothelial cell as proposed for the spleen, either this cell type(s) must be relatively more frequent in the lungs or a higher proportion of it was hit during the acute phase of infection.

Detection of a viral test sequence by PCR does not discriminate between latent infection with a functional genome and abortive infection with a defective genome. The in vivo recurrence is therefore of utmost importance, as it proves that at least a fraction of the detected viral genomes were functional. Interestingly, the risks of recurrence in lungs and spleen correlated with the number of detected viral copies in these two organs.

A promising conclusion from our data is that the murine model of CMV infection and latency is closer to the pathogenesis of human CMV than was previously thought. As a consequence, the focus of interest should be redirected from the spleen to the lungs, the most critical site of human CMV pathology during recurrent infection of immunocompromised patients. The recurrence of virus is most probably not the only parameter in the pathogenesis of interstitial CMV pneumonia (reviewed in references 9 and 35), but it is undoubtedly the prime event. Thus, the finding that the lungs represent a high-risk organ for CMV latency and recurrence adds to the understanding of CMV pneumonia.

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