The Conditions of Primary Infection Define the Load of Latent Viral Genome in Organs and the Risk of Recurrent Cytomegalovirus Disease

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Summary

Recurrence of cytomegalovirus (CMV) from latency is a frequent cause of disease in immunocompromised patients. To date, there is no explanation for the diversity in the clinical manifestations. Primary infection can occur perinatally or later in life, and inevitably results in latent infection. Seropositivity for antibodies against CMV is indicative of latent infection, but is insufficient as a predictor for the risk of recurrence. As a model for this important medical problem, we compared the risks of murine CMV recurrence from latency established after neonatal primary infection and after infection at adult age. The risk of CMV recurrence was high only after neonatal infection. The copy number of latent viral genome in tissues was identified as the key parameter that determines the overall and organ-specific risks of recurrence. Latent CMV burden and risk of recurrence were related to the extent of virus multiplication during primary infection. The presence of latent CMV in multiple organs provides the molecular basis for stochastic events of recurrence in single organs or in any combination thereof. These findings are discussed as a concept of multifocal CMV latency and recurrence. It provides a rationale for the diversity in the clinical outcome of CMV disease.

Human CMV, the human herpesvirus type 5 (HHV-5),[1] is the prototype of the β-subfamily of the herpesviruses (1–3). In general, herpesvirus infections are effectively controlled by the immune system, but without the ultimate clearance of the virus. Instead, the viral genome is retained at specific sites in a latent state out of which reactivation to recurrent infection and recrudescent disease can occur (4, 5). Primary infection, as well as recurrence from latency, leads to overt manifestations of herpesvirus disease in immunologically immature or immunocompromised individuals. Specifically, HHV-5 infection is teratogenic during fetal development and is a major cause of birth defects, its disease manifestations contribute to AIDS, and it is the major viral cause of post-transplantation disease in bone marrow transplantation and solid organ transplantation recipients (for a review see reference 6). Disease can result from recurrence of endogenous HHV-5 in the transplant recipient. In addition, various organs and blood-borne cells from seropositive donors transmit HHV-5 (for a review see reference 7). Manifestations of CMV disease are diverse and range from specific organ to multiorgan diseases. So far, there is no unifying concept that could explain such diversity. However, the target organ of HHV-5 is often the transplanted organ itself. Thus, the incidence of CMV hepatitis was found to be increased in liver transplant recipients as was that of pneumonitis in lung and heart/lung transplant recipients (7). This suggests an identity between the site of recurrence and that of disease manifestation. Antibodies to CMV are indicative of latent infection and serve to define a qualitative risk (8). Ablation of cellular immune control is a prime risk factor for CMV recurrence, but it is apparent that not all risk patients develop CMV disease. The definition of further risk factors is therefore fundamental to any risk prediction.

Murine models of CMV latency and recurrence serve to answer open questions that cannot easily be addressed in clinical research (for a review see reference 9). As with HHV-5, latent murine CMV, the murid herpesvirus 1 (1), can be transmitted by organ transplantation (10, 11), and we have shown in previous work (12) that latency in organs is not explained by latently infected, circulating, or tissue-resident blood-borne cells. The existence of multiple organ sites of CMV latency implies the possibility of independent events of recurrence at these sites.

[1] *Abbreviations used in this paper:* HHV-5, human herpesvirus type 5; IE, immediate early; MEF, mouse embryo fibroblast.

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Data presented here support a concept of multifocal CMV latency and recurrence. Specifically, it is demonstrated that the course of primary infection defines the overall load with latent CMV, and that the copy numbers of latent viral genome in organs correlate with organ-specific risks of recurrence.

Materials and Methods

Infection and Immunization of Mice. Female BALB/c mice, bred and housed at our facility (University of Ulm) under specified pathogen-free conditions, were infected as outlined in the first section of Results with the Smith strain of murine CMV (ATCC VR-194; American Type Culture Collection, Rockville, MD) that was propagated in cell culture and sucrose-gradient purified (12). For testing passive immunization with immune serum, 8-wk-old BALB/c mice were subcutaneously infected with 10^5 PFU of murine CMV at the left hind footpad 3 h after a syngeneic bone marrow transplantation comprising total body γ-irradiation with a single dose of 6 Gy followed by i.v. infusion of 10^6 femoral bone marrow cells from uninfected donors. The passive immunization was performed by i.v. infusion of native serum the day before infection. To serve as the serum donors, C57Bl/6 mice (B6) and B cell–deficient, homozygous μMT/μMT mutants (13) backcrossed to B6 were actively immunized by two i.v. infusions of inactivated murine CMV (10^7 PFU inactivated by 254 nm UV light) within 3 wk. The serum was tested 3 wk after the boost.

Induction and Assay of Recurrent Infection. CMV recurrence was induced by total body γ-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. For plus-minus scoring done 2 wk after the irradiation, recurrent virus was detected from organ homogenates by an in vitro plaque assay with centrifugal enhancement of infectivity on permissive mouse embryo fibroblast (MEF) monolayers essentially as described previously (14), except that no methylcellulose overlay was made and all of the homogenate of each organ was plated to exploit the full sensitivity. Primary plaques were counted after 3–4 d, and negative results were affirmed by the absence of plaques during 10 d of the assay culture. A negative score was given if no plaque was detected after 10 d. For the determination of virus titers, the plaque assay was performed with methylcellulose to preclude secondary plaque formation, and usually 1% of each organ homogenate was then plated, which defines the detection limit of this assay as 100 PFU per organ. It is an inherent problem of virus quantification in organs of seropositive mice that neutralizing antibody contained in the organ homogenate may obscure the assay. Homogenization was therefore done at 4°C, and in a volume diluting neutralizing antibody to below the limit of detection in the plaque reduction assay (see below).

Assay for Neutralizing Serum Antibody. The titer of neutralizing antibody in sera from latently infected mice was determined from the half-maximal plaque reduction in a conventional plaque reduction assay. 10^3 PFU of purified murine CMV in 0.1 ml of plaque assay medium (14) were incubated at 37°C for 2 h in standard vials (Eppendorf, Hamburg, Germany) with 0.1 ml of native serum (or organ homogenate; see above) in appropriate dilutions, followed by the virus plaque assay.

Immunoprecipitation of CMV Proteins. As a source of viral antigens, lysates of infected MEF were prepared 24 h after infection after a 4-h period of metabolic labeling with [^{35}S]methionine in the late phase of viral gene expression. Lysates from CV-1 cells were made 7 h after infection with vaccinia virus wild-type strain Copenhagen or with the vaccinia virus recombinant Vac-gB which expresses the gB gene of murine CMV (15). Procedures for biosynthetical labeling, preparation of cell lysates, and the immunoprecipitation as well as the protein separation on SDS-polyacrylamide (7.5%) gels were done as described previously (15, 16).

Detection of Viral and Cellular DNA. Organs from latently infected mice and blood leukocytes from acutely infected mice were processed for DNA isolation, and specific viral and cellular DNA sequences were amplified by employing PCR based on published methods (17) with certain modifications (12). In brief, a 363-nucleotide sequence was amplified from exon 4 of the murine CMV immediate-early (IE) gene *ie1* (18) by using oligonucleotides IE1.1983 and IE1.2345 as forward and reverse primer, respectively, and oligonucleotide IE1.2135 as the probe for the verification of the identity of the amplification product (12). Primers and probe for detecting the β-actin gene as a cellular gene control have been specified previously (12). Throughout, 3-μg samples of DNA were subjected to 35 cycles of amplification. In the titration of blood leukocytes, the leukocyte DNA was supplemented to 3 μg with certified-negative carrier DNA. The amplification products were analyzed by electrophoresis on 1.4% agarose minigels, Southern blotting, hybridization with the respective ^{32}P-endlabeled probe, and autoradiography.

Samples with all components except template DNA provided the technical negative control. The 12.2 kbp plasmid pIE111, which encompasses genes *ie1* and *ie3* of murine CMV, served as positive controls. The assay sensitivity was determined by titrating 1–1,000 molecules of plasmid pIE111 to 3 μg of carrier DNA from organs of uninfected mice. It was found that 10 copies of the viral gene can generally be detected, that the signal intensities usually increase linearly between 10 and 100 copies, and that the sensitivity of detection is not influenced by the organ source of the carrier DNA (12). Therefore, 10 and 100 molecules of pIE111 mixed to 3 μg of certified-negative spleen DNA were included in the PCR experiments as positive controls. The PCR was evaluated semiquantitatively by ranking the signals as <10, 10, 100, and >100 copies of the viral test sequence.

Results

The Course of Primary Infection Differs with the Infection History. Human CMV is frequently acquired perinatally or during early childhood, and a second epidemiologic wave of primary infection is based on sexual transmission between adults. For both epidemiologic groups, seropositivity for CMV-specific antibodies is indicative of prior infection, and the serological status currently serves as the only criterion for predicting a risk of CMV recurrence in transplantations (8). It is so far unknown whether the history of primary infection leads to differential risks of recurrence within the risk group of seropositives. We have compared the infection of neonates and that of adults in the murine model of CMV infection to investigate the influence of primary infection on the establishment of latency and the risk of recurrence.

The experimental regimen and the time schedule along which acute/persistent primary infection develops into latent infection are schematically outlined in Fig. 1. One group of BALB/c mice, referred to as experimental group N, was infected intraperitoneally with 10^2 PFU of purified murine CMV as neonates on the first day after birth, whereas the second group, referred to as experimental group A, was infected subcutaneously as young adults at the age of 2 mo with 10^5 PFU in the left hind footpad. Duration, extent,

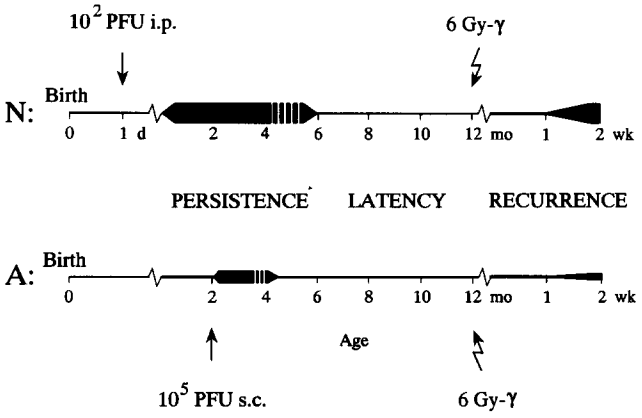


Figure 1. Experimental regimen and time schedule for establishment of latency and induction of recurrence. (Group *N*) Mice infected as neonates. (Group *A*) Mice infected as adults. (*Black cones*) Symbolize duration and productivity of virus replication in salivary glands during persistence and recurrence.

and organ sites of acute virus multiplication differed markedly between these two groups (Fig. 2). In the neonates, CMV caused disease with ~40% mortality occurring typically in the third week. During the period of mortality, high virus titers were observed in a number of organs. Survivors showed a runting diseaselike growth retardation until ~4–6 wk. Around that time, virus multiplication ceased in most tissues except in the salivary glands, where a persistent virus replication continued until 4–6 mo. Beyond 6 mo, the infection had become latent in most individuals with viral DNA detectable by PCR in organs as well as in blood leukocytes (12). In contrast, in adult mice, the infection did not cause mortality or significant morbidity. Virus replication above the detection level was observed only in the salivary glands, and discontinued at this privileged site ~2 mo after infection (Fig. 2). These findings are in good agreement with previous experience (9, 14). In summary, in both models, the salivary glands represent the organ site of persistence, but in group N the acute infection is disseminated, the duration of the persistent phase is longer, and the overall virus productivity is much higher than in group A. We therefore asked next whether these marked differences in primary infection are reflected by differences in the amount of viral genome harbored in organs during latency.

The History of Primary Infection Determines the Tissue Load of Latent CMV. We have shown in previous work that in infected neonates, viral DNA is maintained in blood leukocytes for up to 6 mo after resolution of productive infection, but is then cleared to below the limit of detection by PCR. At that stage of latency, viral DNA remained prevalent in organs, and, in comparison with the spleen, the lungs then proved a major organ site of CMV latency and recurrence (12). We therefore concentrated on the lungs to compare tissue loads of latent viral DNA in experimental groups N and A 1 yr after infection (Fig. 3). To exclude cases of persistent infection or of spontaneous recurrence, the six individuals shown for each group were pretested for absence of infectious virus in tissue fragments from salivary glands, lungs, and spleen. In addition, it was assured for each individual that the viral test sequence, a 363-nucleotide sequence from exon 4 of the IE gene *ie1* (12), was no longer detectable in blood leukocytes by PCR (data not shown). In group N, viral DNA was detected in the lungs of all six mice with uniformly high copy numbers of >100 in 3 μg samples of organ DNA, which equals >200 copies in 10^6 tissue cells (Fig. 3, *top*). In contrast, in group A, the test sample was negative for two out of six mice tested, and in the four positive cases, the copy numbers of the viral sequence were <10 (Fig. 3, *bottom*). A sequence from murine β-actin was amplified in both groups with indistinguishable efficacy from 30 ng samples of lung DNA (Fig. 3).

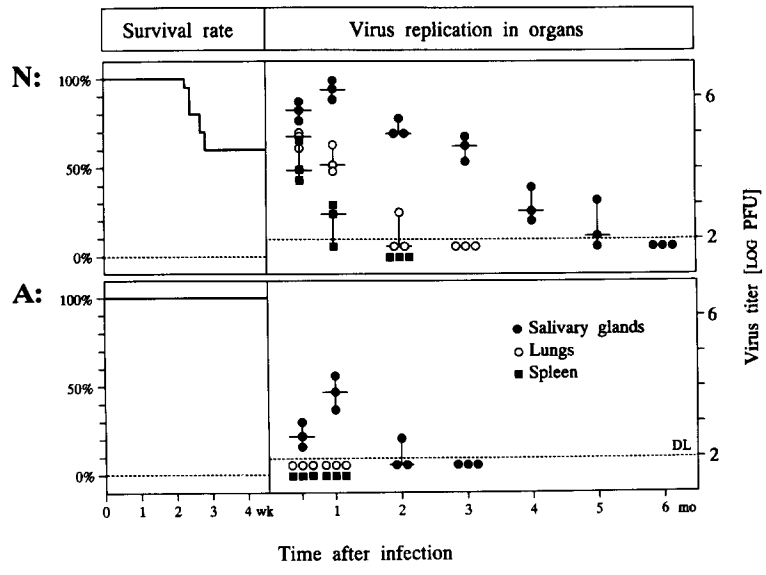


Figure 2. Course of primary murine CMV infection. Mice were infected as indicated in Fig. 1 as neonates (group *N*) or as adults (group *A*). Survival rate (n = 20) and virus titers in organs were monitored. Symbols represent three mice tested individually. (*Vertical bar*) Range; (*horizontal bar*) median value. (*DL*) Detection limit of the virus plaque assay.

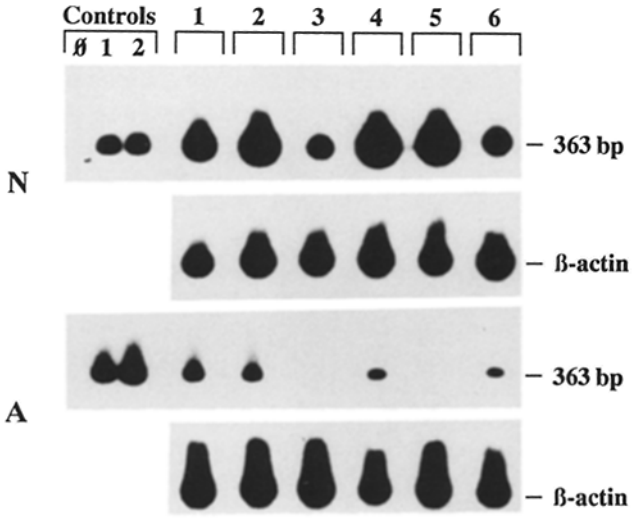


Figure 3. Prevalence of latent viral DNA in the lungs. A 363-nucleotide sequence of exon 4 of the murine CMV immediate-early gene *ie1* was amplified by PCR from 3 μg samples of lung DNA of six individual, latently infected mice (test lanes *1–6*) of experimental groups N and A 1 yr after inoculation, that is, at a time when viral DNA was no longer detectable by PCR in blood. As a control for the technical efficacy of amplification, a sequence from the cellular mouse β-actin gene was amplified from 30 ng of the same lung DNA samples. (Lane ϕ) All reagents except DNA; (control lanes *1* and *2*) 10 and 100 copies, respectively, of plasmid pIE111 mixed to 3 μg of certified-negative carrier DNA derived from the spleen of an uninfected mouse.

This difference was not unique to the lungs. For a representative animal of either group (Figs. 4 and 5 correspond to individuals N2 and A2), the load of latent viral DNA is shown in greater detail by a screening of several organs on the basis of six independently amplified samples per organ. In individual N2, all organs tested were clearly positive, although with marked differences between organs. The overall load was much lower in individual A2. Specifically, spleen

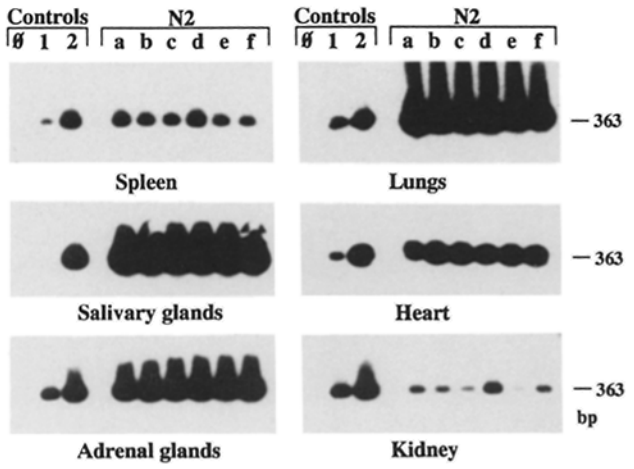


Figure 4. Organ distribution of latent viral DNA after neonatal infection. The viral 363-nucleotide test sequence was amplified from organ DNA of the individual mouse N2. Control lanes as in Fig. 3. (Lanes *a–f*) Six independently amplified 3-μg samples of organ DNA.

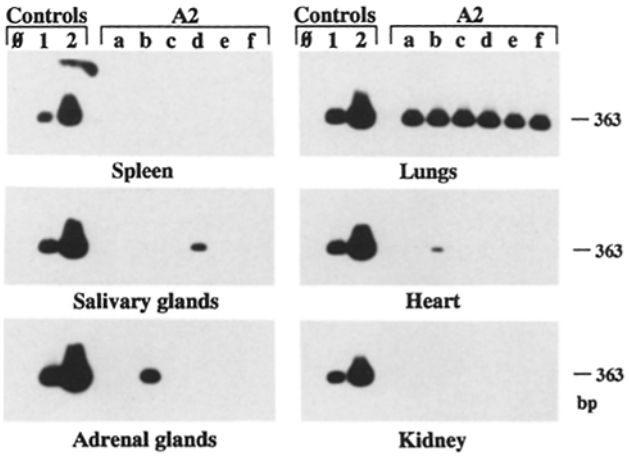


Figure 5. Organ distribution of latent viral DNA after infection at adult age. Legend as for Fig. 4, except that individual A2 was tested.

and kidney were negative at this assay sensitivity, and salivary glands, adrenal glands, and heart were subject to sampling variance with only one of six samples positive, containing <10 copies. However, the viral genome was not cleared from this individual, as the lungs contained ~10 copies of the viral sequence in all six samples tested, which corresponds to ~20 copies per 10^6 tissue cells. Thus, the lungs represent a preferred organ site of murine CMV latency not only after neonatal infection (12), but also after infection of adults. The overall impression from Figs. 3–5 is that the organ distribution of latent viral DNA is qualitatively similar for both protocols of CMV latency, whereas the absolute tissue load is significantly higher in neonatally infected mice.

Segregation Between Acute Productivity and Load of Latent Genome. Whereas the data have indicated that virus dissemination during primary infection is important for a wide organ distribution of the latent viral genome, there is no absolute correlation between the productivity during primary acute/persistent infection and the amount of latent viral genome in a particular organ. The salivary glands represented the site of virus persistence in both groups of latently infected mice. However, the high and long-lasting virus productivity in the salivary glands is not quantitatively reflected by an accordingly high load of latent viral DNA (Figs. 4 and 5). This is particularly obvious in latently infected adults (Fig. 5, individual A2; Fig. 6, individuals A1 and A3), in which the lungs consistently harbored more latent genome than did the salivary glands, even though virus replication during primary infection was barely detectable in the lungs (see Fig. 2).

The Risk of Recurrence Correlates with the Tissue Load of Latent CMV. The amplification of a viral DNA sequence from host tissues by PCR does not discriminate between defective and functional genomes. The proportion of functional latent genomes capable of initiating recurrent infection may differ between organs. We therefore induced recurrence in vivo (12) to test whether the amount of viral DNA detected by PCR can be used as a predictor for the risk of recurrence (Table 1). The experiment was done 1 yr after infection, when in-

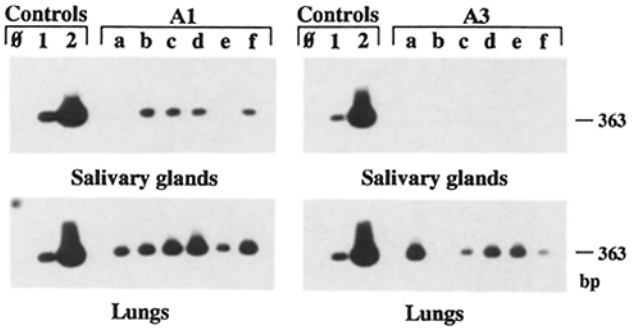


Figure 6. Comparison of latent CMV load in salivary glands and lungs after infection at adult age. Legend as for Fig. 4, except that individuals A1 and A3 were tested.

fectious virus was no longer detectable in any organ (n = 30; 0/30 positive). The incidence of induced recurrence was compared for experimental groups N and A, and was monitored for three organs separately. A negative score was given, if no virus was detected. Titers for positive scores ranged from 10^2 to 10^4 PFU.

In organs of mice infected as adults, recurrent virus was not detectable, except in the lungs of two individuals out of 30 tested (Table 1, group A). In contrast, there was a highly significant recurrence in mice infected as newborns (Table 1, group N). Interestingly, recurrent virus was rarely found simultaneously in all three organs tested. Instead, discrete patterns of virus distribution were observed (Table 1, patterns II–VIII). The cumulative incidence of recurrence in the three organs tested was 63% in this experiment, whereas the incidence for any particular organ was lower (summing up of patterns II–VIII). As a consequence, screening of only one organ for recurrent CMV underestimates the overall incidence of recurrence, and, accordingly, the determined incidence is only a minimum estimate, as possible recurrence in further organs remained untested. The organ-specific incidence was high for salivary glands and lungs, and lower for the spleen. Thus, overall and organ-specific incidences of recurrence reflected the respective differences in the amount of viral DNA detected by PCR.

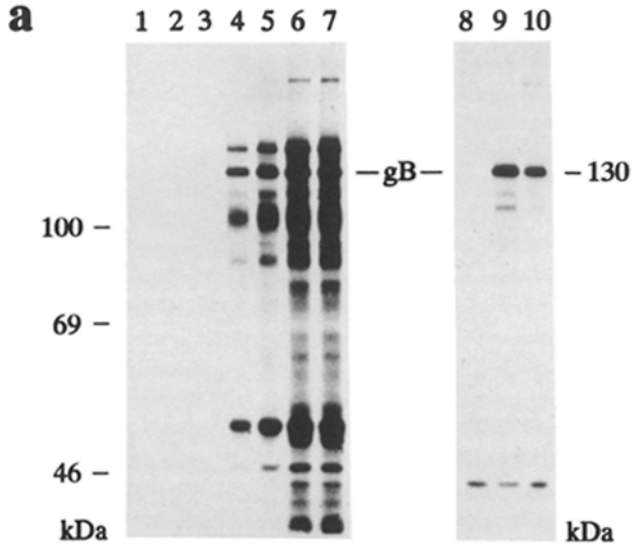
Evidence for Stochastic Multifocal Recurrence. The demonstration of latent viral DNA in multiple organs and the observed discrete patterns of recurrence with more frequent detection of recurrent virus in those organs that harbored latent CMV in high copy number, strongly suggested that recurrence is a stochastic event that occurs independently in different organs with a likelihood defined by the number of latent viral genomes in tissue. The alternative would be that recurrent CMV originates from a single site of latency and disseminates to multiple organs where it may be detected with different sensitivity based on differential productivity of the tissue cells. As the salivary glands represent the privileged site of CMV replication, cases of positive salivary glands corresponding to negative lungs and spleen (Table 1, pattern II) are not a strong argument against dissemination. However, along the same line of argument, the frequent cases of positive lungs corresponding to negative salivary glands (Table 1, pattern III) are not compatible with dissemination. If recurrence is a stochastic multifocal event, cases of coincident recurrence in two or more organs must occur with an incidence predicted by the laws of statistics. This is indeed the case for the data in Table 1. When tested by Fisher's exact probability test, the number of observed double-positive events for all three possible pairs between three organs is in good agreement with the hypothesis of independent distribution (p-values ranging from 0.25 to 0.55).

Neutralizing Antibody Does Not Prevent Recurrence. Antiviral antibody must be discussed as an additional parameter that

Table 1. *Incidences of Induced Murine CMV Recurrence*

Pattern	Detection of recurrent CMV in organs			Incidence group N	Incidence group A
	Salivary glands	Lungs	Spleen		
I	–	–	–	11/30	28/30
II	+	–	–	5/30	0/30
III	–	+	–	6/30	2/30
IV	–	–	+	2/30	0/30
V	+	+	–	3/30	0/30
VI	+	–	+	2/30	0/30
VII	–	+	+	0/30	0/30
VIII	+	+	+	1/30	0/30
II–VIII group N	11/30 (36.7%)	10/30 (33.3%)	5/30 (16.7%)	19/30 (63.3%)	
II–VIII group A	0/30	2/30 (6.7%)	0/30		2/30 (6.7%)

CMV recurrence was induced by 6 Gy of γ-irradiation in groups of 30 latently infected BALB/c mice inoculated as newborns (group N) or as adults (group A). Virus was detected by plaque assay at day 14 after the irradiation.



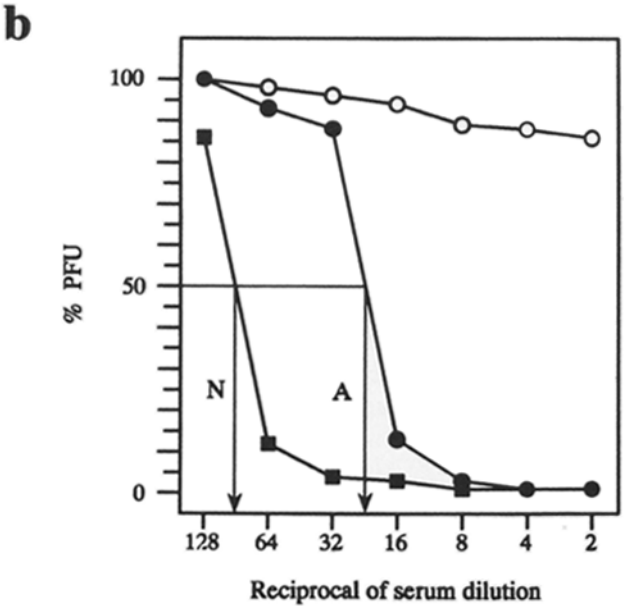
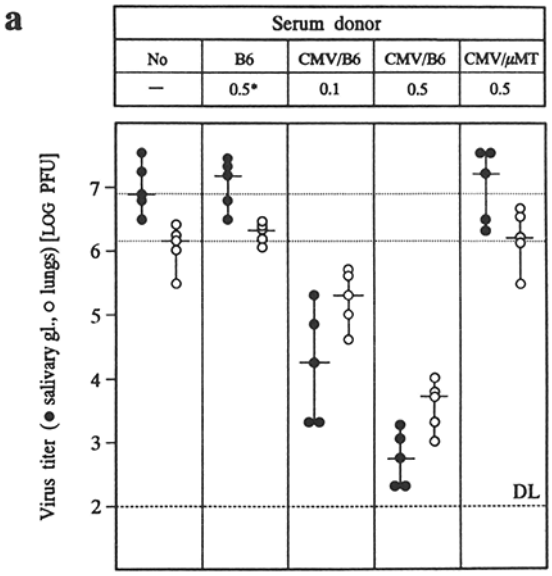


Figure 7. Antiviral specificity and virus neutralization capacity of immune sera. Serum was pooled from five mice each of experimental groups N and A shortly before the recurrence experiment (see Table 1). (*a*) (*Left*) Immunoprecipitation of viral late-phase proteins. (Lane *1*) Nonimmune serum tested with lysate from infected MEF; (lanes *2* and *3*) sera of groups N and A, respectively, tested with lysate from uninfected MEF; (lanes *4* and *5*) two independent immunoprecipitations from infected MEF with group N serum; (lanes *6* and *7*) with group A serum, accordingly. (*Right*) Immunoprecipitation of the murine CMV glycoprotein B (*gB*). (Lane *8*) Immunoprecipitation with group A serum from lysate of CV-1 cells infected with wild-type vaccinia virus, strain Copenhagen; (Lanes *9* and *10*) immunoprecipitation with group N and group A serum, respectively, from lysate of CV-1 cells infected with the recombinant vaccinia virus Vac-gB. (*b*) Plaque reduction assay determining the titer of virus neutralizing antibodies in group N (■) and group A (●) sera. Serum pooled from uninfected, age-matched mice served for control (○). Counted plaque numbers are normalized.



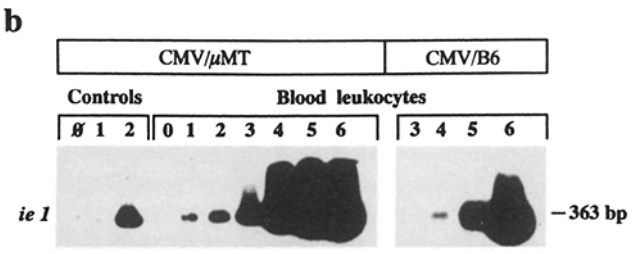


Figure 8. Reduction of hematogenic virus dissemination by immune serum. (*a*) Virus titers were determined in organs of γ-irradiated (6 Gy), bone marrow reconstituted BALB/c recipients 2 wk after s.c. infection (10^5 PFU). The day before infection, the mice were passively immunized by i.v. transfer of 0.1 or 0.5 (*) ml of serum derived from primed B6 mice (*CMV/B6*) or from primed B cell–deficient μMT/μMT mutant mice (*CMV/μMT*). Groups with no serum transfer (*No*) or transfer of serum from unprimed B6 mice (*B6*) served as negative controls. Symbols represent five individual mice per group. The median values are marked by a horizontal bar. (*b*) Frequency of blood leukocytes carrying viral DNA. 1 mo after serum transfer (0.5 ml) and infection, the viral 363-nucleotide test sequence was detected by PCR in graded numbers of leukocytes. (Lane *ϕ*) No template DNA; (control lanes *1* and *2*) 10 and 100 copies, respectively, of plasmid pIE111 supplemented to 3 μg with carrier DNA; (test lanes *0–6*) DNA from 10^0 to 10^6 blood leukocytes, respectively, supplemented to 3 μg with carrier DNA at cell numbers of $<10^6$.

may influence the risk of recurrence and account for the difference between adult and neonatally latent mice. We have therefore determined the serological status for both groups (Fig. 7), qualitatively by immunoprecipitation of viral proteins (Fig. 7 *a*) and quantitatively by the functional test of virus neutralization (Fig. 7 *b*). There were differences in the capacity of both sera for precipitating viral proteins from lysates of infected MEF, with group A serum precipitating more proteins (Fig. 7 *a*, *left*). However, serum from neonatally infected mice was equally potent in precipitating the 130-kD glycoprotein B (*gB*) of murine CMV (15) from lysates of cells infected with the recombinant vaccinia virus Vac-gB (Fig. 7 *a*, *right*). This finding is relevant, as the human homolog,

the gB of HHV-5, represents a major antigen for recognition by neutralizing antibodies (19), and vaccination with Vac-gB protects mice against lethal challenge infection (Rapp, M., and U. H. Koszinowski, manuscript in preparation). Moreover, the serum of group N proved to have a fourfold higher titer of neutralizing antibody (Fig. 7 *b*). Thus, the incidence of induced recurrence was higher in organs of neonatally infected mice in spite of a higher titer of neutralizing antibodies in serum.

Evidence that Serum Antibody Controls Virus Dissemination. The finding that recurrent infection led to discrete patterns of positive organs (see Table 1) requires an explanation. If γ-irradiated (6 Gy), unprimed mice are infected intravenously, 1–2 PFU are sufficient to cause disseminated infection in all susceptible organs. In contrast, latently infected mice are resistant against i.v. superinfection with $\leqslant 10^5$ PFU even when cellular immunity by CD8 T cells is abrogated by in vivo anti-CD8 treatment in addition to the γ-irradiation (data not shown). These observations, along with the known protective antiviral capacity of passive immunization with immune serum (20), suggest that antiviral antibody was the factor that prevented the dissemination of recurrent infection from the organ sites of recurrence to unaffected organs in the experiment of Table 1.

To exclude an involvement of putative other immune serum components, serum from CMV-primed B cell–deficient μMT mutant mice (13) was compared with serum from primed parental B6 mice with respect to the efficacy in blocking hematogenic dissemination of CMV upon prophylactic i.v. transfer into nonimmune recipient mice (Fig. 8). The day after the serum transfer, the recipients were γ-irradiated, bone marrow reconstituted, and infected subcutaneously with murine CMV. The bone marrow transplantation was required to overcome the lethal bone marrow aplasia and blood panleukopenia caused by CMV (21). Antibody-containing serum from parental B6, but not antibody-free serum from the μMT mutant, reduced virus titers in target organs in a dose-dependent manner (Fig. 8 *a*). Moreover, the frequency of blood cells that carried viral DNA detected by PCR 1 mo after infection was reduced from 1/10 to $1/10^4$ by parental B6 serum (Fig. 8 *b*). In conclusion, antiviral antibody can limit hematogenic dissemination of CMV.

Discussion

For clinical CMV in transplantations, a potential risk of recurrence is defined qualitatively by the serological status of transplantation donor and recipient (8). However, CMV-specific antibody is only indicative of latent CMV infection, and the low positive predictive value of seropositivity for recurrent CMV infection and disease does not justify prophylactic antiviral treatment. Primary CMV infection can occur throughout life, and is usually not diagnosed, because it passes without overt symptoms. Thus, except in rare cases of a clinical CMV anamnesis, the time point of primary infection is unknown for the individual patient, and seropositives are viewed in case statistics as a uniform risk group. It is therefore open to question, whether the individual risk differs with the history of primary infection, and which parameters could be used as more reliable predictors for the risk of recurrence.

In this paper, we have compared the risks of murine CMV recurrence in experimental models designed to represent two more distant cases in the infection epidemiology of human CMV, namely the infection of neonates and adults. The data have shown that the risk of recurrence differs largely between the two groups. Neonatal infection is associated with a high risk of recurrence, whereas the risk is low when primary infection occurred at adult age.

Specifically, four major conclusions can be drawn from the experiments: (*a*) the extent of virus replication and dissemination during primary infection, which is high after neonatal infection and low after infection of adults, determines the overall burden of latent viral DNA in organs; (*b*) the copy number of latent viral DNA in organs correlates with the overall and organ-specific incidences of in vivo recurrence; (*c*) recurrence is a stochastic, focal event that occurs independently in different organs; and (*d*) antiviral antibody cannot preclude recurrence at the organ sites of latency, but can limit virus dissemination.

In their inquiry into the mechanisms of α- and γ-herpesvirus latency, Roizman and Sears (4) proposed a determinative role of primary infection, and specifically, an importance of the copy number of latent viral genome for recurrence. These principles are now demonstrated for a β-herpesvirus. CMV latency differs from herpes simplex virus latency by its wide organ distribution. The presence of latent CMV in multiple organs provides the molecular basis for recurrence from multiple organs. A cellular site of CMV latency has not yet been precisely identified. For murine CMV latency in the spleen, there is evidence for a location of latent viral DNA in the stromal compartment (22, 23), and the sinusoidal lining cell is discussed as a candidate (22). The low frequency of latently infected cells revealed by PCR (12) has so far precluded an analysis of the affected cell type in other organs. It is a promising aspect of our results that knowledge of the latently infected cell and of the viral copy number per cell is not a prerequisite for predicting the risk of recurrence, as the average viral copy number relative to tissue DNA, estimated from PCR amplification of a single essential viral gene, can substitute for that information. This may be of practical importance with respect to a possible prognostic medical application. According to our findings, a high copy density in a tissue predicts a low threshold for recurrence. It should be possible to test that prediction at least for the donor organ–associated risk in transplantation patients.

At first glance, the finding that the viral copy number in latently infected salivary gland tissue does not quantitatively reflect the gross productivity during acute and persistent infection argues against the proposed role for viral replication in the establishment of latency. A discrepancy was particularly obvious in mice infected as adults, in which latent viral genome was barely detectable in salivary glands, even though virus had been replicating there in the persistent phase for several weeks. There is an explanation for this finding. During persistent infection in the salivary glands, virus replication is confined to a particular cell type, the acinar glandular epi-

thelial cell (24, 25). As termination of persistence is associated with necrosis of this producer cell (24), another cell type must be proposed as the cellular site of latency.

The observation of stochastic recurrence in various organs (Table 1) may help to explain another unsolved problem in the prediction of CMV disease. Interstitial pneumonia is the most frequent manifestation of human CMV after bone marrow transplantation (26, 27), but, on occasion, other manifestations predominate and range from specific organ diseases to multiorgan disease. We predict from our results in the experimental model that patients with a high overall burden of latent CMV are at risk of a multifocal recurrence in many organs, which can resemble disseminated CMV disease after primary infection of an immunocompromised host. In contrast, in patients in which the overall burden of latent CMV is low, recurrence is more likely to occur only in single organs, with a preference for organs with high copy numbers of latent viral DNA. If, for instance, recurrence takes place by chance in the lungs, interstitial pneumonia can be the result, whereas recurrence in salivary glands leads to virus secretion without overt CMV disease.

There is considerable interest in antibody prophylaxis of posttransplant CMV disease as well as in vaccination trials (28). However, from the clinical experience, the benefit of such approaches is controversial (29). That antiviral antibodies do not preclude recurrence has always been obvious from the epidemiologic data, according to which seropositivity was even used as a qualitative risk predictor. The model has reproduced this, but it proposes a beneficial role for antibody in the prevention of disseminated infection after a focal recurrence.

In essence, the concept of multifocal cytomegalovirus latency and recurrence provides a rationale for the as yet poorly understood diversity in the disease manifestations of CMV. In may prove useful as a basis for further studies.

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