The r144 Major Histocompatibility Complex Class I-Like Gene of Rat Cytomegalovirus Is Dispensable for both Acute and Long-Term Infection in the Immunocompromised Host

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The rat cytomegalovirus (RCMV) r144 gene encodes a polypeptide homologous to major histocompatibility complex class I heavy chains. To study the role of r144 in virus replication, an RCMV r144 null mutant strain (RCMVΔr144) was generated. This strain replicated with efficiency similar to that of wild-type (WT) RCMV in vitro. Additionally, WT RCMV and RCMVΔr144 were found not to differ in their replication characteristics in vivo. First, the survival rate was similar among groups of immunosuppressed rats infected with either RCMVΔr144 or WT RCMV. Second, the dissemination of virus did not differ in either RCMVΔr144- or WT RCMV-infected, immunosuppressed rats, either in the acute phase of infection or approximately 1 year after infection. These data indicate that the RCMV r144 gene is essential neither for virus replication in the acute phase of infection nor for long-term infection in immunocompromised rats. Interestingly, in a local infection model in which footpads of immunosuppressed rats were inoculated with virus, a significantly higher number of infiltrating macrophage cells as well as of CD8+ T cells was observed in WT RCMV-infected paws than in RCMVΔr144-infected paws. This suggests that r144 might function in the interaction with these leukocytes in vivo.

The genomes of cytomegaloviruses (CMVs) comprise approximately 180 open reading frames (ORFs) (12, 22), several of which are homologous to genes of the host organism. Most of these ORFs are suspected to interfere with the immune system of the host and encode putative chemokines and chemokine receptors. In addition, genes homologous to mammalian major histocompatibility complex (MHC) class I genes have been identified within the genomes of two CMV species: human CMV (HCMV) (1) and murine CMV (MCMV) (22). In this report, we present the identification and characterization of a third herpesvirus gene putatively encoding an MHC class I homolog, the rat CMV (RCMV) r144 gene.

Identification, cloning, and sequence analysis of the RCMV r144 gene. Previously, it was shown that the majority of RCMV genes are colinear with genes of both HCMV and MCMV (2–5, 29, 30). However, the genes of HCMV and MCMV encoding MHC class I homologs (UL18 and m144, respectively) are localized within dissimilar regions of their respective genomes (12, 22). Since previously described RCMV genes were found to share more sequence similarity with the corresponding genes of MCMV than with those of HCMV (2–5), we hypothesized that a putative RCMV gene homologous to MHC class I genes would be located in a genomic region similar to that of MCMV m144. Accordingly, we focused on a 20-kb region of the RCMV genome spanning from the EcoRI fragment to the XbaI P fragment (Fig. 1A) (20). As shown in Fig. 1A, an ORF (r144) having significant similarity to the MCMV m144 gene was identified within this region. The RCMV r144 ORF has a length of 963 bp and potentially encodes a 321-amino-acid polypeptide with a predicted molecular mass of 36 kDa. The sequence of this polypeptide shows 30 and 19% similarity with the amino acid sequences encoded by MCMV m144 and HCMV UL18, respectively. The predicted amino acid sequence of the r144-encoded protein (gpr144) was compared with sequences from a representative set of mammalian MHC class I polypeptides as well as with the amino acid sequences derived from MCMV m144 and HCMV UL18 (gpm144 and gpUL18, respectively). The sequences of these polypeptides were included in a CLUSTAL W multiple alignment (Fig. 1B). The alignment shows four cysteine residues to be conserved between gpr144, gpm144, gpUL18, and mammalian MHC class I polypeptides. These conserved cysteines, which might play a role in disulfide bridge formation (7), are located at positions 111, 139, 176, and 235 of gpr144. Within the gpr144 sequence, three putative N-linked glycosylation sites are present. One of these sites, at positions 95 to 97, is positionally conserved between gpr144, gpm144, and mammalian MHC class I proteins. Based on the alignment shown in Fig. 1B, the putative r144 gene product, as well as the UL18- and m144-encoded proteins, can be assigned a domain structure similar to that previously determined for human HLA-A2. The HLA-A2 protein was found to consist of six domains, as follows: (i) a putative N-terminal leader sequence, (ii) an α1 domain, (iii) an α2 domain, (iv) an immunoglobulin-like α3 domain, (v) a putative transmembrane α-helix region, and (vi) a short intracellular region (7). It was previously shown that gpm144 shows a considerable deletion within its putative α2 region compared to the α2 region of mammalian MHC class I molecules (11). Interestingly, gpr144 has a similar deletion within its putative α2 domain, whereas the corresponding region of gpUL18 comprises several small insertions relative to mammalian MHCs (11) (Fig. 1B). The α1 and α2 regions of mammalian class I proteins were demonstrated to form a groove for binding small antigenic peptides to be presented on
the cell surface (6). The loss of sequences within the corresponding regions of gpm144 may account for the inability of this protein to form a complex with peptides, in contrast to HCMV gpUL18 (11). Considering the sequence similarity between gpr144 and gpm144, gpr144 might similarly be unable to bind peptides. In order to show the relationship between the sequences of all known virus-encoded MHC class I homologs and those of several mammalian MHC class I proteins, a phylogenetic tree was constructed (Fig. 1C). This tree shows that the mammalian MHC class I molecules represent the most conserved group of sequences, whereas the virus-encoded homologs are relatively divergent. Most notably, the phylogenetic distances between the gpUL18 sequence and the gpr144, the gpm144, and the poxvirus MHC-like sequences (gpMC1080R and gpMC2080R) are similar. This suggests that the incorporation of a UL18-like gene into an ancestral genome of HCMV on the one hand and the incorporation of an r144- or m144-like gene in an ancestral genome of both RCMV and MCMV on
transcription of ORFs neighboring r144 (data not shown). Notably, transcripts of r144 could not be detected by Northern blotting either in RCMV- or in RCMVΔr144-infected REF. Similarly, transcription of m144 has not been demonstrated in MCMV-infected cells.

**Replication characteristics of RCMVΔr144 in vitro.** To compare the replication characteristics of RCMVΔr144 with those of WT RCMV in vitro, we infected three different cell types with these viruses and determined the percentage of infected cells at various time points after infection, in a manner similar to that described previously (2, 4). In addition, the amount of infectious virus that was produced by each cell type was investigated. The cell types tested included REF, rat heart endothelium cell line 116 (31), and monocyte and macrophage cell line R2 (14). We found that the percentage of infected cells did not differ significantly between WT RCMV- and RCMVΔr144-infected cells, irrespective of the cell type. Moreover, no significant differences between WT and recombinant viruses in the virus titers produced by each cell type were observed (data not shown). These data indicated that r144 is not essential for RCMV replication in these cell types in vitro. Similar results have previously been reported for both the HCMV strain with a deletion of the UL18 gene (8) and the MCMV strain with a deletion of the m144 gene (15).

**In vivo RCMVΔr144 infection.** Infection of immunocompetent rats with RCMV generally results in asymptomatic infections in which the virus can be detected almost exclusively in the salivary glands (9). By contrast, RCMV infection of immunocompromised animals usually leads to disease in which viral replication can be observed in many organs and tissues of the rats (2, 4, 9, 27). Consequently, in order to investigate the role of r144 in the pathogenesis of RCMV disease, we infected rats with either WT RCMV or RCMVΔr144 after the induction of immunosuppression by total-body Röntgen irradiation. In an initial experiment, two groups of 4-week-old, immunosuppressed rats (five animals per group) were inoculated with 10^6 PFU of either WT RCMV or RCMVΔr144. The number of surviving rats in each group was monitored until day 28 postinoculation (p.i.). Surprisingly, no significant difference in the survival rate of groups of rats infected with either WT RCMV or RCMVΔr144 was observed (data not shown). As expected, the results illustrated the integrity of the genomes of both WT and recombinant virus (data not shown).

Our results with the virus strain with a deletion of the r144 gene are in contrast to the data reported by Farrell and co-workers (15), who showed that an MCMV strain with a deletion of the homolog of r144, m144, was severely restricted in replication in visceral organs compared with WT MCMV during the acute phase (days 2 to 6) of infection in mice. In addition, it was demonstrated by in vivo depletion studies that...
TABLE 1. Dissemination of WT RCMV and RCMVΔr144 in various organs of infected, immunocompromised rats

<table>
<thead>
<tr>
<th>Organ</th>
<th>floated</th>
<th>No. of positive organs/total no. of organs tested by</th>
<th>PCR on day 330 p.i.</th>
</tr>
</thead>
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<tr>
<td>Salivary gland</td>
<td></td>
<td>WT</td>
<td>Δ</td>
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<tr>
<td>Spleen</td>
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<td>0/5</td>
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<tr>
<td>Kidney</td>
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<td>Thymus</td>
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<td>Lymph tissue</td>
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<tr>
<td>Aorta</td>
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<tr>
<td>Leukocytes</td>
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<td>0/5</td>
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<tr>
<td>Bone marrow</td>
<td></td>
<td>ND</td>
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*a* Ratios are the number of organs found to be positive for either WT RCMV or RCMVΔr144 (Δ)/*the total number of organs tested (one organ per rat). Immune peroxidase assays (IPOX) and plaque assays were carried out as described previously (2, 4).

ND, not determined.

natural killer (NK) cells are responsible for the attenuated phenotype of the m144 knockout virus (15). Based on these findings, Farrell et al. proposed that MCMV employed the m144 gene product to mimic cellular MHC class I molecules (of which the cell-surface expression is downregulated during infection) by inhibiting the NK cell response. Thus, the early clearance of MCMV-infected cells would be prevented (15). A similar function has also been suggested for the HCMV UL18 phenotype of the m144 knockout virus (15). Based on these findings, Farrell et al. proposed that MCMV employed the r144 gene product to serve as a decoy to evade immune surveillance, as was similarly proposed for MCMV gpm144 and RIE3R2 (5).

In a similar spectrum of organs of infected rats, virus DNA could be detected in the spleen of four of five RCMVΔr144-infected rats, whereas spleen tissue from three of three WT RCMV-infected rats was PCR negative. This difference is, given the variation in viral DNA load among infected rats (data not shown), likely due to differences in the local immune response, which was not investigated further.

Dissemination of RCMVΔr144 on day 330 p.i. Although differences between WT and recombinant virus were not seen during the acute phase of infection, we considered the possibility that disruption of the RCMV r144 gene might have an effect on long-term persistent or latent infection of rats. We therefore investigated dissemination of RCMV and RCMVΔr144 at a late stage of infection. Thus, two groups of 6-week-old, immunosuppressed rats (five animals per group) were infected with 105 PFU of either RCMV or RCMVΔr144. On day 330 p.i., total cellular DNA was purified from various tissues and organs of the rats using an XTRAX DNA extraction kit (Gull Laboratories, Salt Lake City, Utah). Then the DNA samples were subjected to a sensitive, single-tube, nested PCR that enables amplification of part of the major immediate-early region of the RCMV genome (3). The PCR mixtures (50 μl) contained 1 μg of target DNA, 0.05 μM concentrations of primers RIE3F (5'-CCA GAG TGA CGT TGC AGA TGT TGG AAA TCA-3'); nucleotides 3425 to 3454 of the sequence assigned GenBank accession no. AF046125) and RIE3R2 (5'-GGT CAC GAC CCT GCT GCC GTG TAG GT-3'; complement of nucleotides 3719 to 3744 of the sequence assigned GenBank accession no. AF046125), 1 μM concentrations of primers RIE4F (5'-ATG AAA TGG TGA TGA GAT-3'; nucleotides 3461 to 3748 of the sequence assigned GenBank accession no. AF046125) and RIE4R (5'-CTT CTA GTG ATT TGG CAT-3'; complement of nucleotides 3686 to 3707 of the sequence assigned GenBank accession no. AF046125), 100 μM concentrations of each deoxynucleoside triphosphate, 1.25 U of HotStar Taq DNA polymerase (Qiagen, Leusden, The Netherlands), and HotStar Taq DNA polymerase buffer (Qiagen). Amplification was performed with a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer, Nieuwerkerk aan de IJssel, The Netherlands), which was programmed to incubate the samples for 15 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 70°C, and 30 s at 72°C and 25 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Finally, the tubes were incubated for 5 min at 72°C. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. This procedure enabled detection of 1 to 10 copies of genomic RCMV DNA per μg of organ tissue (1 to 10 RCMV genome copies per 2.4 × 105 cells) (data not shown). As shown in Table 1, DNA from both WT RCMV and RCMVΔr144 was detected in a similar spectrum of organs of infected rats. Virus DNA was detected most consistently in the salivary glands and the liver (Table 1), irrespective of whether rats were infected with WT RCMV or RCMVΔr144. Interestingly, virus DNA could be detected in the spleen of four of five RCMVΔr144-infected rats, whereas spleen tissue from three of three WT RCMV-infected rats was PCR negative. This difference is, given the variation in viral DNA load among infected rats (data not shown), likely due to differences in the local immune response, which was not investigated further.

Infection of rat footpads with RCMVΔr144. Previously, Perrones et al. (21) described a model for local RCMV infection in which the footpad of immunosuppressed rats is inoculated with virus. As a result of this infection, severe macro- and microscopic pathology can be seen, including paw thickening,
ballooning of endothelial cells, adherence of polymorphonuclear and mononuclear cells to the endothelial surface, and infiltration of inflammatory cells into the perivascular area (21). We employed this model to compare WT RCMV and RCMVΔr144 in the pathology induced by local infection with these viruses. Groups of 6-week-old, immunosuppressed rats (five animals per group) were injected subcutaneously with 106 PFU of either WT RCMV or RCMVΔr144 in the dorsum of the right hind paw, in a manner similar to that described previously (21). As a control, supernatant from mock-infected REF cultures was injected in the left hind paw of the rats. The thickness of the rat paws was measured daily, as described by Persoons et al. (21). In a separate, similar experiment, rats were sacrificed at either day 4, day 8, or day 15 after infection and sections (4 μm) of the paws were investigated for the presence of infiltrating leukocytes by using leukocyte subset-specific monoclonal antibodies. As described previously (21), infection of rat footpads with RCMV resulted in thickening of the paws. Similar macroscopic alterations were observed after infection with RCMVΔr144, whereas changes in the mock-infected paws were not detected (data not shown). Interestingly, we found significant differences between WT RCMV and RCMVΔr144 in the number of infiltrating leukocytes in the infected paws. At day 15 p.i., a significantly lower number of macrophages was detected in RCMVΔr144-infected paws than in RCMV-infected paws (Fig. 3A). In accordance with this, significantly lower numbers of cells expressing VLA-4 (Fig. 3B), LFA-1 (data not shown), and CD4 (Fig. 3C) were detected in RCMVΔr144-infected paws than in WT RCMV-infected paws at day 15 p.i. VLA-4, LFA-1, and CD4 can be expressed on the surface of various subsets of leukocytes, including monocytes and macrophages. Also, a small but significant difference between recombinant virus- and WT virus-infected paws in the number of CD8+ cells (means ± standard errors of the means, 111 ± 11 and 152 ± 9, respectively) was observed at day 15 p.i. Although CD8 can be expressed on both NK cells and a subset of T lymphocytes, it is likely that the CD8+ cells do not represent NK cells, since in the number of NK cells RCMVΔr144- and WT RCMV-infected paws did not significantly differ (Fig. 3D). It is possible that the observed differences in leukocyte influx resulted from differences in replication between RCMV and RCMVΔr144. However, both viruses were found to have similar replication characteristics in the rat paws, as judged by similar viral DNA loads as well as similar expression levels of RCMV early proteins (data not shown). Taken together, our data indicate that a higher number of macrophages and CD8+ T cells infiltrate in WT virus-than in RCMVΔr144-infected rat paws. This suggests that the r144-encoded protein might play a role, either directly or indirectly, in the interaction with macrophages and CD8+ T cells rather than NK cells. Interestingly, our findings may provide support for the hypothesis of Leong et al. that viral MHC class I homologs may be more important in affecting monocyte and dendritic cell function than in affecting NK cell function (19). This hypothesis was based on results of Cosman and coworkers (13), who found that the ICMV UL18 gene product can interact with a membrane receptor, designated ILT2 (24) or LIR-1 (13), which is predominantly expressed on monocytes and B lymphocytes. Since ILT2/LIR-1 is expressed on only a minor subset of NK cells (13), the physiological significance of the interaction of gpUL18 with this receptor on NK cells is unclear. Leong et al. hypothesized that the interaction of gpUL18 with ILT2/LIR-1 on monocytes or dendritic cells could suppress IL12 production, which would limit the secretion of gamma interferon by NK cells, thereby altering the early immune response (19). Such a mechanism could also explain the severely restricted replication of the MCMV m144 knockout virus in vivo (15).

Whether the RCMV r144-encoded protein is actually expressed in vitro and in vivo, and whether this protein is able to interact with macrophages and CD8+ T cells, will have to be investigated in future studies. These studies should also include the characterization of RCMVΔr144-derived virus strains in which the disrupted r144 gene has been repaired, since we cannot rule out the possibility that the effects that were observed in the local infection model are due to adventitious mutations at sites of the RCMV genome other than the r144 gene. Nevertheless, it is likely that the identification of a putative cellular receptor for gpUL144 may prove crucial in the elucidation of the role of this protein during RCMV infection.

**Nucleotide sequence accession number.** The sequence of the RCMV genome spanning from EcoRI O to XbaI H, which contains the r144 ORF (Fig. 1A), and the predicted amino acid sequence derived from r144 have been deposited in the GenBank database under accession no. AF133339.
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