Mixed infection with multiple strains of murine cytomegalovirus occurs following simultaneous or sequential infection of immunocompetent mice

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As with human cytomegalovirus (HCMV) infection of humans, murine CMV (MCMV) infection is widespread in its natural host, the house mouse Mus domesticus, and may consist of mixed infection with different CMV isolates. The incidence and mechanisms by which mixed infection occurs in free-living mice are unknown. This study used two approaches to determine whether mixed infection with MCMV could be established in laboratory mice. The first utilized two naturally occurring MCMV strains, N1 and G4, into which the lacZ gene was inserted by homologous recombination. The lacZ gene was used to track recombinant and parental viruses in simultaneously coinfected mice. In the second approach, a real-time quantitative PCR (qPCR) assay was used to detect viral immediate-early 1 (ie1) gene sequences in mice successively coinfected with G4 and then with the K181 MCMV strain. In both systems, mixed infection was detected in the salivary glands and lungs of experimentally infected mice. MCMV-specific antibody in sera and G4 IE1-specific cytotoxic lymphocyte responses in the spleens of twice-infected mice did not prevent reinfection. Finally, the prevalence of mixed infection in free-living mice trapped in four Australian locations was investigated using real-time qPCR to detect ie1 DNA sequences of N1, G4 and K181. Mixed infection with MCMVs containing the G4 and K181 ie1 sequences was detected in the salivary glands of 34.2% of trapped mice. The observations that mixed infections are common in free-living M. domesticus and are acquired by immunocompetent mice through simultaneous or successive infections are important for vaccine development.

INTRODUCTION

The prevalence of human cytomegalovirus (HCMV) is widespread in many human populations (Britt & Alford, 1996) where primary infection is asymptomatic for most individuals (Sissons & Carmichael, 2002). In principle, new viral genotypes may appear in the infected host either through mutation or reinfection with a different viral strain. Infection of individuals with more than one HCMV genotype was first thought to occur only in those with altered immunity such as AIDS patients (Drew et al., 1984; Gerna et al., 1992; Spector et al., 1984), transplant recipients (Chou, 1986) and pregnant women (Arav-Boger et al., 2002; Huang et al., 1980; Shen et al., 1993). However, mixed infection of healthy individuals may frequently occur. Multiple genotypes of the glycoprotein B (gB) gene were detected in organ and blood samples collected from 25 people at necropsy (Meyer-König et al., 1998), and also in women who regularly attended clinics for sexually transmitted disease (Chandler et al., 1987). The mechanism by which mixed CMV infection occurs in healthy individuals is unknown. The presence of multiple strains of a virus in the infected host has significant implications for the design of vaccines where numerous immunologically distinct viral strains may confound vaccination attempts.

To understand further the phenomenon of mixed infection with multiple CMV variants, we have utilized murine CMV (MCMV) infection of inbred laboratory and free-living mice
as a model for human infection with HCMV. HCMV and MCMV are members of the subfamily *Betaherpesvirinae* of the family *Herpesviridae* (van Regenmortel et al., 2000). Infection of laboratory mice with MCMV is used in many experimental systems investigating CMV pathogenesis and disease, as CMV infections are species-specific (Kim & Carp, 1971; Osborn, 1981). Similar to most HCMV infections, MCMV infection is asymptomatic in healthy mice when acquired via natural routes of transmission (Faroway et al., 2002). MCMV is ubiquitous in free-living mice (Mus domesticus) trapped in rural areas in Australia (Booth et al., 1993; Moro et al., 1999; Singleton et al., 1993; Smith et al., 1993) and other countries (Gardner et al., 1974; Mannini & Medearis, 1961; Plummer, 1973). Furthermore, mixed infections with multiple, genetically variable MCMV strains have been detected in free-living mice by using restriction fragment length polymorphism (RFLP) analysis (Booth et al., 1993).

We were interested in determining whether mixed infection with MCMV could be established experimentally in BALB/c mice through either simultaneous or asynchronous inoculation with two different viral strains, in the context of vaccine development. Mice were initially inoculated simultaneously with a wild-type strain and an alternate recombinant virus. Mice were then inoculated with two viral strains. Finally, in order to investigate the extent of mixed infection with MCMV in free-living mouse populations, we used real-time qPCR to detect a number of MCMV sequences after mice were serially inoculated with two viral strains. In order to investigate the extent of mixed infection with multiple, genetically variable MCMV strains synchronously with a wild-type strain and an alternate recombinant virus, we were interested in determining whether mixed infection with MCMV in free-living mouse populations, we used real-time qPCR to detect a number of MCMV sequences after mice were serially inoculated with two viral strains. In order to investigate the extent of mixed infection with multiple, genetically variable MCMV strains synchronously with a wild-type strain and an alternate recombinant virus, we were interested in determining whether mixed infection with MCMV in free-living mouse populations, we used real-time qPCR to detect a number of MCMV sequences after mice were serially inoculated with two viral strains.

**METHODS**

**Mice.** Highly inbred BALB/c (H2b) adult (8 week old) female mice were purchased as specific-pathogen-free from the Animal Resources Centre (Murdock, Western Australia) and maintained under minimal disease conditions. Sentinel mice were free of a set of murine pathogens including MCMV following routine testing. All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia.

**Virus.** Dr A. Scalzo (University of Western Australia, Australia) provided the N1 and G4 isolates of MCMV, which were originally obtained from the salivary glands of free-living mice (M. domesticus) trapped at Nannup (N1) or Geraldton (G4) in Western Australia (Booth et al., 1993). Dr. D. Lang (Duke University, NC, USA) originally provided K181 (Chalmer et al., 1997), a laboratory strain of MCMV considered to be a virulent variant of the Smith strain (Misra & Hudson, 1980).

**Cells and virus stock production.** The M210B4 cell line was obtained from the ATCC. Generation of M210B4 cells for the production of tissue culture virus (TCV) stocks was as described previously (Lutarewych et al., 1997). Confluent cells in tissue culture flasks were infected with 1 × 10⁶ p.f.u. MCMV in RPMI 1640 medium with 2 % fetal calf serum (FCS), 20 μM L-glutamine and 40 μg gentamicin ml⁻¹ under conditions of centrifugal enhancement (Hudson, 1988) at 800 g for 30 min at 37 °C. Flasks were incubated at 37 °C with 5 % CO₂ until 100 % cytopathic effect was evident. Infected cells were scraped into the supernatant and samples were centrifuged at 11 000 g for 30 min at 4 °C. The pellet was resuspended in 5 ml RPMI with 2 % FCS, frozen to −80 °C and thawed to release virus from cells. Cellular debris was removed by centrifugation at 300 g for 5 min at 4 °C and supernatant was then collected and stored at −80 °C.

**Recombinant virus.** The N1lacZ and G4lacZ recombinant viruses were produced using homologous recombination techniques, which required the co-transfection of viral DNA of the N1 and G4 strains with linearized pON427⁺ plasmid. Professor E. Morcarski (Stanford University, California, USA) provided pON427⁺, which contains a lacZ gene cassette inserted between two HpaI sites in the ie2 gene on the HindIII L fragment of K181. Insertion of this gene cassette resulted in the deletion of a 79 bp fragment of ie2 as described previously (Manning et al., 1992). The ie2 gene is non-essential for virus replication both in vitro and in vivo (Cardin et al., 1995; Manning & Morcarski, 1988; Manning et al., 1992). A purified clonal population was acquired using three rounds of plaque purification in conjunction with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) to identify cells infected with a recombinant MCMV expressing β-galactosidase. RFLP analysis of recombinant and parental viral DNA with the HindIII restriction enzyme (Promega) followed by Southern blot analysis confirmed that the lacZ gene was located on the same HindIII fragment as the ie1 and ie2 genes (data not shown). RT-PCR confirmed that transcripts of the ie1 and m131/129 open reading frames of the recombinant viruses were identical to their parental strains, indicating that the expression of genes surrounding the site of insertion was not disrupted (data not shown).

**Plaque assays.** Organs were homogenized to form 10 % extracts by using sterilized pestles (Kontes) in 1 ml RPMI with 2 % FCS. Samples were clarified by centrifugation at 800 g for 20 min at 4 °C and supernatants were stored at −80 °C. The plaque assay was used to quantify infectious virus present in organ extracts in duplicate as described previously (Allan & Shellam, 1984), except that M210B4 cells were used to detect infectious MCMV. Viral titres are expressed in p.f.u. salivary gland g⁻¹ (limit of detection ≤500 p.f.u. g⁻¹), where negative samples were given values of 500 p.f.u. g⁻¹ (the limit of detection) in order to calculate geometric means.

**Detection of β-galactosidase expression.** Infected cell samples or salivary gland extracts were serially diluted in RPMI with 2 % FCS and 200 μl was used to infect confluent monolayers of M210B4 cells for 1 h at 37 °C with 5 % CO₂. The inoculum was aspirated and 1 ml RPMI with 0-7 % carboxymethyl-cellulose and 2 % FCS was added to each well. When plaque formation became visible, cell monolayers were fixed with gluteraldehyde (0-5 % in PBS), and β-galactosidase expression was detected after incubation with PBS supplemented with 0-5 mg X-Gal ml⁻¹ for 2 h at 37 °C.

**In vivo cytotoxic T-cell assay.** Splenocytes from naive mice were used as target cells, after the lysis of erythrocytes with 0-15 M NH₄Cl. Target cells were pulsed with the G4 IE1 (YPMFNPPS) or K181 IE1 (YPHFMTNPL) peptides by incubating 10⁶ cells with 1 ng peptide for 90 min at 37 °C and then labelled with 0-025 μM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes). A second population of splenocytes was labelled with 0-25 μM CFSE. Target cells (CFSE⁺) and control cells (CFSE⁻) were then mixed at a ratio of 1:1, and 5 × 10⁵ cells were adoptively transferred into the peritoneal cavity of nude mice.
transferred into infected mice. Splenocytes were collected from recipi-
ent mice after 18 h and the CFSE-labelled populations were de-
tected by flow cytometry (FACScan; Becton Dickinson) and an-
alysed using CellQuest software (Becton Dickinson). Specific cyto-
toxic effector function in the spleen was determined by a reduction in
target (CFSE<sup>lo</sup>) cells relative to control cells (CFSE<sup>hi</sup>).

**Mouse trapping procedures.** *M. domesticus* were live-trapped
using Longworth (Jacob et al., 2002) or Elliott traps (Moro et al.,
2003) from free-living populations located in four Australian
locations including: Walpeup (Victoria: 35° 13′ S 149° 48′ E),
Gungahlin (Australian Capital Territory: 35° 5′ S 142° 12′ E),
Boullanger Island (Western Australia: 30° 19′ S 115° 00′ E) and
Macquarie Island (an oceanic subantarctic island south of Tasmania:
54° 30′ S 158° 57′ S). Blood was collected from mice by cardiac
puncture and serum was extracted by centrifugation at 800 g for
2 min. Salivary glands were collected from trapped mice following
autopsy. The numbers of mice trapped at the Boullanger Island,
Macquarie Island, Walpeup and Gungahlin sites were 27, 40, 38 and
12, respectively (total = 117).

**MCMV isolation and RFLP analysis.** The method used for
purifying MCMV isolates from salivary glands of *M. domesticus* and
generation of viral DNA was as described previously (Booth et al.,
1993), except that M210B4 cells were used for the generation of viral
DNA. Approximately 2 μg MCMV DNA was digested for 4 h at
37 °C with 12 U EcoRI (Promega) and then electrophoresed on a
0.8%-agarose gel for 16 h at 70 V.

**Real-time qPCR.** We recently described a real-time qPCR system
(GeneAmp 5700 Sequence Detection System, Applied Biosystems) to
detect *ie1* sequences of the N1 and G4 strains of MCMV (Faroway et al.,
2005). In this study, we have also used real-time qPCR to
detect the *ie1* sequence of K181. These strains are distinguished by
variable nucleotide sequences located within the immunodominant
H2<sup>L</sup>/CTL epitope encoded within the *ie1* gene (Lyons et al., 1996).
The reverse primer (N1/G4/K181 R) was used for the detection of all
viral sequences, whilst the forward primers N1 F and G4/K181 F
were used for the detection of N1 or G4 and K181 *ie1* viral
sequences, respectively (Table 1). The probes, N1, G4 and K181
(Table 1), were used in conjunction with the primers to detect the
N1, G4 and K181 *ie1* sequences, respectively, to produce a 126 bp
product. PCR conditions including primer and probe concentra-
tions, reagent mix, thermal cycling conditions and negative controls
were as described previously (Faroway et al., 2005). The rodent gly-
ceraldehyde-3-phosphate dehydrogenase (GAPDH) primer and
probe system (Applied Biosystems) was used as an internal standard
for viral DNA extraction. Plasmids containing the *HinIII* L frag-
ment of the N1, G4 or K181 strains were used as positive controls
and standards (Faroway et al., 2005) as the *HinIII* L fragment of
MCMV encodes the *ie1* gene (Keil et al., 1987). Calculated standard
curves had correlation coefficients (r) that ranged from 0.93 to 1.00
(Table 1). Data were further processed to determine the number of
viral genome copies (g genome)<sup>-1</sup>. The limit of detection of this assay,
with the detection of 1 genome per reaction, was 3.5 × 10<sup>3</sup> MCMV
genomes g<sup>-1</sup>.

**ELISA.** Antibody specific for MCMV was detected in serum samples
using ELISA as described previously (Lawson et al., 1988). MCMV
antigen preparations were obtained following the infection of
M210B4 cells (as for TCV stocks) with K181 and at 100% cyto-
pathic effect, viral antigen was collected from the supernatant by
centrifugation at 18 000 g for 2 h at 4 °C (Beckman L8-70M, SW41
rotor). Sera collected from naïve BALB/c mice were used as negative
controls. Sera collected from BALB/c mice inoculated intraperitoneally
(i.p.) three times, at 2 week intervals, with 2 × 10<sup>4</sup> p.f.u.
K181 and bled 2 weeks after the third inoculation were used as posi-
tive controls. Samples were considered positive at the dilution where
absorbance values were three standard deviations greater than the
mean of the absorbances achieved by the negative controls. Antibody
titres are the reciprocal of this dilution.

**Table 1. Primers, probes and standard curves for real-time qPCR**

<table>
<thead>
<tr>
<th>Primer/probe name</th>
<th>Primer/probe sequence (5′–3′)</th>
</tr>
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<tbody>
<tr>
<td>N1 F</td>
<td>TACCGCTGTCTCAATCTGAGTTT</td>
</tr>
<tr>
<td>G4/K181 F</td>
<td>TACGGCTGTCTGAGCTGAGTTT</td>
</tr>
<tr>
<td>N1/G4/K181 R</td>
<td>CCCTAGTACCTCATCCAGACTCTCT</td>
</tr>
<tr>
<td>N1 probe</td>
<td>ACTTAGACCTCAGCCCCCTAATCTAGG</td>
</tr>
<tr>
<td>G4 probe</td>
<td>ACCCACAAGTCTAGCCCCCTAGCTAGG</td>
</tr>
<tr>
<td>K181 probe</td>
<td>ACCCACAAGTCTAGCCCCCTAATCTAGG</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Standard curve</th>
<th>r&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>y = −1.18ln(x) + 38.6</td>
<td>0.867</td>
</tr>
<tr>
<td>G4</td>
<td>y = −1.40ln(x) + 39.5</td>
<td>0.978</td>
</tr>
<tr>
<td>K181</td>
<td>y = −1.23ln(x) + 39.4</td>
<td>0.940</td>
</tr>
<tr>
<td>N1</td>
<td>y = −1.44ln(x) + 38.3</td>
<td>1.000</td>
</tr>
<tr>
<td>G4</td>
<td>y = −1.58ln(x) + 41.1</td>
<td>0.966</td>
</tr>
<tr>
<td>K181</td>
<td>y = −1.39ln(x) + 40.6</td>
<td>0.955</td>
</tr>
</tbody>
</table>

*F, Forward primer.
†R, Reverse primer.
‡§Plasmids containing the N1, G4 and K181 *HinIII* L fragments were diluted in nuclease-free water
containing DNA extracted from the salivary glands‡ or lungs§ of naïve adult BALB/c mice to create
standard curves for the conversion of real-time qPCR C<sub>T</sub> scores (y) to copy number of viral genomes (x).
The C<sub>T</sub> score is the cycle at which a statistically significant increase in the magnitude of the fluorescent
signal was first detected.
**Statistical analyses.** Viral titres and ELISA results were compared between treatments using one-way analysis of variance (followed by Tukey’s post-hoc analysis) or the Student’s *t* test as appropriate. Values reported represent mean ± standard error unless stated otherwise.

**RESULTS**

**Mixed infection can be established in the salivary glands of mice simultaneously infected with two MCMV strains**

To determine whether mixed infection with more than one genetic variant of MCMV could be established experimentally BALB/c mice were inoculated simultaneously with two

![Image](https://www.microbiologyresearch.org/)

**Fig. 1.** Viral titres in the salivary gland after simultaneous coinfection with a mixture of two MCMV strains. Wild-type (WT) and recombinant (REC) β-galactosidase-expressing viruses were identified by staining mouse embryonic fibroblast monolayers infected with salivary gland homogenates from MCMV-infected mice with X-Gal, as shown in (a). Adult female BALB/c mice were i.p. infected with a total of 4 × 10^4 p.f.u. Salivary glands were removed from mice (*n* = 6) at 14 (b) and 35 (c) days post-infection and tested for the presence of infectious MCMV by plaque assay in conjunction with staining for β-galactosidase expression by recombinant MCMVs (open symbols). Wild-type viral titres are shown as closed symbols. Lines represent geometric means (limit of detection < 500 p.f.u. g⁻¹). Fractions shown on the top right of each box are the number of mice per infection group with both recombinant and wild-type viruses detected in salivary gland samples. Note that rMCMV-lacZ stably expresses β-galactosidase *in vivo* as all plaques isolated from the salivary glands of mice infected with either N1lacZ or G4lacZ turn blue upon staining with X-Gal.
obtained where both wild-type and recombinant viruses were detected in the salivary glands of at least one mouse per treatment group at both 14 and 35 days post-infection (unpublished data). These results demonstrate that a mixed infection can be readily established in the salivary glands of BALB/c mice simultaneously inoculated with two MCMV strains.

The use of recombinant viruses encoding β-galactosidase has several disadvantages. Recombinant viruses can be less competitive than wild-type viruses due to the immunological burden of the transgene. In this study, titres of the wild-type virus G4 were significantly greater than the recombinant G4lacZ at 14 days post-infection in the salivary glands of mice coinfected with G4 and G4lacZ (Fig. 1b, \( P = 0.006 \)). In addition, viral plaques and thus β-galactosidase expression were not detected from organ samples of mice with low-level persisting or latent infections. We chose to use a specific real-time qPCR assay to circumvent these problems and have used this assay previously to detect the presence of the \( i e 1 \) sequences of the N1 and G4 MCMV strains in the salivary glands of infected mice (Farroway et al., 2005). Additional primers and probes for the detection of the \( i e 1 \) sequence of the K181 strain were developed. The specificity of the primers and probes (Table 1) for the detection of the N1, G4 or K181 \( i e 1 \) sequences was tested by using alternate strain-specific primers and probe sets to detect the presence of the \( i e 1 \) sequence of a particular strain in salivary gland samples, which were collected from BALB/c mice infected i.p. with \( 2 \times 10^5 \) p.f.u. of the viral strain. Salivary glands from these mice contained infectious virus as determined by the plaque assay. Primers and probes were specific for the detection of the appropriate viral sequences only (data not shown).

**Mixed infection can be detected in the salivary glands and lungs of mice asynchronously infected with two MCMV strains**

Real-time qPCR was used to determine if a mixed infection could be established in vivo after serial inoculations with two MCMV strains. BALB/c mice were injected i.p. with \( 4 \times 10^5 \) p.f.u. G4 or mock infected with diluent. After 28 days, mice were reinfected i.p. with either \( 4 \times 10^4 \) p.f.u. G4, K181 or mock infected with diluent. There were four treatment groups (\( n = 6 \) mice per treatment): G4 + G4, G4 + K181, G4 + diluent and diluent + diluent. These treatments were chosen to determine whether the K181 strain could reinfect mice already infected with the G4 virus. An interval of 28 days between infections was chosen to avoid the peak of the IE1-specific CTL response, which is maximal 6–8 days after i.p. infection with either G4 or K181, although significant CTL activity was detected as late as 50 days post-infection (see Fig. 3). The salivary glands and lungs were removed from mice 35 days after the initial inoculation and tested for infectious virus by plaque assay and for the \( i e 1 \) DNA sequences of G4 and K181 using the real-time qPCR assay.

**Fig. 2.** Detection of MCMV strains in tissues, CTL responses and serum antibody titres following serial coinfection with G4 and K181. Adult female BALB/c mice were infected i.p. with \( 4 \times 10^5 \) p.f.u. G4 and then 28 days later with the same dose of G4 or K181. In some treatments, diluent (RPMI with 2% FCS) was used to inoculate mice. Organs were removed from mice (\( n = 6 \)) 35 days after the primary inoculation. Viral titres in the salivary glands as detected by the plaque assay are shown in (a) where lines represent geometric means. Salivary glands (b) and lungs (c) were tested for the presence of G4 (closed symbols) and K181 (open symbols) \( i e 1 \) DNA sequences by real-time qPCR. At 34 days post-infection, target cells were pulsed with G4 IE1 peptide (d) or K181 IE1 peptide (e) and adoptively transferred into three mice from each group, respectively. After 18 h, lysis of targets by splenocytes was determined by FACS analysis. MCMV-specific antibody titres are shown for serum collected at 35 days post-infection (f) as detected by ELISA. Data are from individual mice infected with G4 + G4 (circles), G4 + K181 (triangles), diluent + G4 (diamonds) or diluent + diluent (crosses). For (b)–(f) lines represent arithmetic mean values with negative samples not shown.

Neither infectious virus nor viral DNA was detected in the salivary glands or lungs of negative control mice (diluent only, Fig. 2). Even though the number of mice containing infectious MCMV varied from two to four (Fig. 2a), there was no difference in viral titres in the salivary glands when all groups were compared (\( F = 1.18, P = 0.34 \)). Secondary infection with the same or a different MCMV strain did not reduce viral titres, with the salivary glands of four of six mice treated with G4 + G4 or G4 + K181 positive for infectious virus (Fig. 2a). Infectious virus was not detected in the lungs of mice from any treatment group. G4 and K181 \( i e 1 \) sequences were simultaneously detected in the salivary
Specific CTL responses were measured in the spleens of infected mice using an in vivo assay, which tested the ability of splenocytes to lyse adoptively transferred target cells pulsed with either the K181 or G4 IE1 peptides. The advantage of this assay over the tetramer-based and gamma interferon intracellular staining methods is that the capacity of viral-specific CTL to lyse IE1 targets is measured in vivo. The K181 and G4 nona-peptides contain an immunodominant, H2Kd-restricted CD8+ T-cell epitope located within the ie1 gene (Del Val et al., 1988; Lyons et al., 1996; Reddehase et al., 1989). In mice initially infected with the G4 virus, spleen cells recognized G4 IE1 targets (Fig. 2d) and not K181 IE1 targets, with the exception of one mouse from the G4 + G4 treatment group (Fig. 2e). Lysis of G4 IE1 (Fig. 2d) but not K181 IE1 (Fig. 2e) target cells was detected in the spleens of mice from the G4 + K181 treatment. Thus, in twice-infected mice there were limited cross-reactive CTL responses. Cross-reactive CTL responses were detected 7 days after i.p. inoculation of BALB/c mice with 4 x 10^3 p.f.u. K181 or G4 (Fig. 3) but were undetectable at 20 days post-infection.

Sera obtained from mice 35 days after the initial inoculation were tested by ELISA for MCMV-specific antibodies. There was a significant increase in antibody titres in the sera of mice sequentially infected with two viruses as compared with mice infected with only one virus (Fig. 2f, F=111.9, d.f. = 17, P < 0.001; Tukey’s post-hoc, P < 0.001). The significant boost in antibody titres following secondary infection confirmed that a memory response was elicited in mice from the G4 + G4 and G4 + K181 treatments. Despite the boost in MCMV-specific antibody titres, a mixed infection with G4 and K181 was still detected in the salivary glands (Fig. 2b) and lungs (Fig. 2c) of some mice sequentially infected with G4 and then K181.

We also examined viral loads in the salivary glands and lungs 128 days post-primary infection, at a time when virus was expected to be latent or cleared from the infected mice. At this time, in mice sequentially infected with G4 and then K181 (G4 + K181), we detected G4 ie1 DNA in the salivary glands (6.6 ± 6.6 x 10^2 viral copies g^-1) and lungs (4.1 ± 0.3 x 10^2 viral copies g^-1) but not K181 ie1 DNA. Thus, a mixed infection consisting of the G4 and K181 viruses may not be able to persist. Interestingly, G4 IE1-specific (3.8 ± 4.4% lysis of G4 IE1 targets) but not K181 IE1-specific CTL responses were detected in mice from the G4 + K181 treatment at any time post-infection, suggesting that long-lived IE1-specific CTL responses require viral persistence.

A mixed infection with two or more MCMV genotypes is commonplace in free-living Australian wild mice

The extent of mixed infection in free-living M. domesticus population is unknown, although Booth et al. (1993) detected a mixed infection in three mice from two Australian sites by using RFLP analysis. We have also used RFLP analysis to detect mixed infection in a free-living mouse trapped from Gunghalin near Canberra, Australia. MCMV variants were isolated by plaque purification from the salivary glands of the mouse (C4). Four genetically distinct MCMV isolates were identified in this mouse (Fig. 4, lanes 1–4). However, RFLP has limitations for determining the extent of mixed infection with MCMV as it is time-consuming, expensive and restriction sites may not be located within highly variable genes. Instead, we used the real-time qPCR assay to detect ie1 sequences, defined in this study as corresponding with genotypes of the N1, G4 and K181 strains of MCMV, in the salivary glands of free-living mice trapped at four Australian locations as this technique is inexpensive, rapid and repeatable.

The G4 and K181 ie1 genotype sequences were detected in mice from all sites and concomitantly detected in the salivary glands of mice trapped at each location, where the prevalence of mixed infection ranged from 27.5 to 66.7% (Fig. 5a). In total, mixed infection was detected in the salivary glands of 34.2% (40 of 117) of trapped mice. While copy numbers of each viral genotype detected in the salivary glands was variable (Fig. 5b), there was no difference in the number of viral genomes of G4 or K181 genotypes (g salivary gland)^-1 from mice trapped in any location (F= 1.73, d.f. = 233, P=0.10). However, mice from Boullanger Island tended to have reduced loads of viral DNA than mice.
from the other tested sites. G4 or K181 ie1 genotypes were detected in the salivary glands of 100% of mice trapped on Macquarie and Boullanger Islands, respectively (Fig. 5a). Interestingly, the N1 ie1 sequence was not detected in the salivary glands of any trapped mouse. The prevalence of MCMV infection using real-time qPCR, ELISA and plaque assay after results for all locations were pooled (n = 117) and was 94.0, 59.0 and 34.2%, respectively.

**DISCUSSION**

We have shown that mixed infection with more than one MCMV ie1 genotype is common in free-living mice from various Australian locations. Mixed infections were also experimentally established in the salivary glands and/or lungs of BALB/c mice either simultaneously or asynchronously infected with two viral strains. We have also documented coinfection of wild-type and recombinant lacZ-expressing viruses of the same MCMV strain (for example, N1+N1lacZ) as reported in previous studies (Grzimek et al., 1999; Saederup et al., 1999). *In situ* hybridization techniques were used recently to determine that dual infection of host cells occurs more frequently than statistically predicted (Cicin-Sain et al., 2005). Mixed infections can be established via the intranasal route of infection, a more natural inoculation route of infection (Mocarski & Kemble, 1996), which may mimic the natural route of MCMV transmission (Mannini & Medearis, 1961; Osborn, 1981) after inhalation or ingestion of saliva containing virus shed from the salivary glands of an infected mouse (Plummer, 1973). Mixed MCMV infections may thus be acquired by free-living *M. domesticus* by either the simultaneous or sequential transmission of MCMV strains.

Previous investigations of mixed HCMV infection of human populations have indicated that immune status may contribute towards the incidence of mixed infection (Arav-Boger et al., 2002; Chou, 1986; Drew et al., 1984; Gerna et al., 1992; Huang et al., 1980; Shen et al., 1993; Spector et al., 1984). Similarly, the immune status of wild mice may also

**Fig. 4.** EcoRI restriction profiles of MCMV isolates purified from the salivary glands of a mouse captured in Gunghalin (ACT, Australia). Lanes 1–8 contain digests of isolates C4A, C4B, C4C, C4D, C7A, N1, G4 and K181, respectively. Lane 9 contains 1 μg 1 kb plus DNA ladder (Gibco-BRL).

**Fig. 5.** Detection of N1, G4 and K181 ie1 DNA sequences by real-time qPCR in the salivary glands of free-living mice trapped at four Australian locations. In (a) the prevalence of each ie1 sequence and in (b) the number of MCMV genomes (g salivary gland)−1 are shown (means are shown by lines). The prevalence of mice infected with both G4 and K181 are shown (G4+K181). Black bars/squares, Boullanger Island (BI); hatched bars/circles, Macquarie Island (MI); white bars/triangles, Gunghalin (GU); grey bars/diamonds, Walpeup (WA).
influence the incidence of mixed infection. Biological factors that modify the immune status of mice include viral and parasitic loads (Smith et al., 1993), breeding stress and environmental factors that alter food abundance (Teo et al., 1991). Mixed infection might occur in stressed individuals during extreme events, such as mouse plagues (Booth et al., 1993). However, the incidence (34-2%, 40 of 117) of mixed infection cannot be attributed solely to the immune status of the mouse populations tested. Instead, mixed infection is probably a feature of MCMV infection in the free-living mouse. We hypothesize that mixed infection may promote the persistence of MCMV in free-living mice through complementation, where the presence of a number of different viral strains would increase genetic variation and thus enhance the fitness of the coinfecting viruses as a population. In addition, MCMV may have an increased ability to evade immune responses of the host, due to enhanced variation within immune targets of the coinfecting viruses.

The frequent detection of the G4 and K181 ie1 genotypes among wild house-mice may indicate an increased capacity of these viruses to persist and transmit among their hosts in comparison to viruses with the N1 ie1 genotype. The lack of detection of the N1 ie1 sequence was an unexpected result and this strain is probably a unique variant. We have preliminary data (S. Nikolovski & S. Gorman, unpublished results), which indicates that the G4 strain has an enhanced capacity to transmit from infected mice to their cage-mates compared with N1. As also demonstrated by Wheat et al. (2003), real-time qPCR is more sensitive than plaque assay and ELISA for detecting MCMV infection. Following the immunization of BALB/c mice with a TCV stock of G4, Morley et al. (2003) could not detect viral titres in mice challenged 90 days later with a salivary gland-derived stock of K181. The technique used in our experiments, real-time qPCR, was a more sensitive method of detection, with mixed infections identified at early times post-infection. However, there are limitations associated with using this real-time qPCR system to detect mixed infection with MCMV. It is uncertain whether differences detected within ie1 reflect real strain differences between coinfected MCMV strains. Lyons et al. (1996) defined five groups of isolates with naturally occurring variant sequences at the IE1 L^2-restricted CTL epitope. The G4, N1 and K181 IE1 sequences correspond to groups 2, 4 and 5, and without incorporating the additional sequence variations of groups 1 and 3 in this study we may have underestimated the extent of mixed infection. In addition, the gB gene may be an appropriate target for the identification of specific MCMV variants as it contains a highly variable antibody-binding sequence (Xu et al., 1996), and has been used to detect mixed infection in man with HCMV (Meyer-König et al., 1998).

We have shown that mice already infected with MCMV can be reinfected with a new strain, despite viral-specific immune responses, indicating that sterilizing immunity to MCMV does not occur. Pre-existing antibody and CTL responses did not prevent reinfection, even though MCMV-specific antibody titres were increased in serum following secondary infection. Furthermore, the absence of K181 IE1-specific CTL responses together with the resolution of polyclonal CTL responses 20 days after infection with G4 probably enabled the dissemination of K181 into the salivary glands and lungs. Prior infection with G4 induced G4 IE1-specific CTL responses, but these did not prevent reinfection with the heterologous strain K181. Lyons et al. (1996) found that G4-primed CTLs could lyse both heterologous K181 IE1 peptide-pulsed targets and homologous G4 IE1 peptide-pulsed targets. While our data confirms this, we only found this phenomenon to occur early after MCMV infection. It is possible that dissemination of K181 occurred via the infiltration of mononuclear phagocytes of the peritoneal cavity into the salivary glands and lungs, without further virus replication. It is uncertain whether a productive infection with K181 was established, as we have not used reverse transcription techniques to detect viral RNA of early- or late-expressing genes (e.g. gB). CTL are required for the clearance of acute MCMV infection in BALB/c mice, as adoptive transfer of sensitized-CD8^+ T cells limits MCMV dissemination, prevents tissue destruction and protects mice from lethal MCMV disease (Reddehase et al., 1984, 1987, 1988). Recently, it has been shown that adoptive transfer of memory CD8^+ T cells specific for the L^2-restricted IE1 epitope were highly protective in mice (Pahl-Seibert et al., 2005) and similar data were obtained with regard to HCMV IE1-specific CD8 responses in human transplant recipients (Bundel et al., 2005). In addition, CMV infection occurs in a substantially lower proportion of seropositive than seronegative transplant recipients (Singh et al., 2005) and maternal immunity is associated with a reduced risk of congenital CMV disease (Fowler et al., 2003). Nonetheless, our data indicates that it may be very difficult to prevent reinfection in the face of an established CTL response in immunocompetent mice. In immunocompetent people, immunity to HCMV does not entirely prevent clinical disease after reinfection with a new strain of virus. Naturally infected HCMV seropositive and Towne strain vaccinated seronegative volunteers received a low-passage Toledo strain as the challenge virus (Adler et al., 1995; Plotkin et al., 1989). Although most volunteers who were either naturally seropositive for HCMV or who had been vaccinated with Towne resisted clinical disease more effectively than naive individuals, clinical disease was still observed in some immunized individuals.

A further reason for preventing reinfection is the growing evidence from immunocompromised transplant patients that the presence of multiple HCMV gB variants correlates with higher virus loads, an increased prevalence of HCMV disease and a higher rate of graft rejection (Coaquette et al., 2004). To be effective, it may be necessary for future HCMV vaccines to induce durable polyclonal CTL immune responses such as those observed early during infection, to prevent reinfection with different viral strains. This will be a major challenge in vaccine development.
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