Forced lytic replication impairs host colonization by a latency-deficient mutant of murine gammaherpesvirus-68

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A regulated switch between latent and lytic gene expression is common to all known herpesviruses. However, the effects on host colonization of altering this switch are largely unknown. We deregulated the transcription of the gene encoding the major lytic transactivator of murine gammaherpesvirus-68, ORF50, by inserting a new and powerful promoter element in its 5′ untranslated region. In vitro, the mutant virus (M50) transcribed ORF50 at a high level and showed more rapid lytic spread in permissive fibroblast cultures, but in vivo, the M50 virus showed a severe deficit in latency establishment, with no sign of the infectious mononucleosis-like illness normally associated with wild-type infection. Although a low level of M50 viral DNA was detectable by PCR in spleens, replication-competent virus could not be recovered beyond 10 days post-infection. The M50 virus was also attenuated in immunocompromised mice. Thus a gammaherpesvirus unable to shut off lytic cycle gene expression showed severely restricted host colonization.

INTRODUCTION

A regulated switch between latent and lytic gene expression is fundamental to the life cycle of all herpesviruses. Essentially, this is a switch between a stable, relatively quiescent genome and a transcriptional programme dedicated to rapid virion assembly and release. The gammaherpesviruses use latency both to provide a reservoir of viable genomes and to drive the proliferation of infected lymphocytes. The latter process is central to normal host colonization (Coleman et al., 2003). It probably also underlies the predisposition of gammaherpesvirus-infected individuals to lymphoid tumours. Disrupting gammaherpesvirus latency thus represents an opportunity both to reduce the extent of infection and to prevent disease.

The expression of a key viral transactivator, for example BZLF-1 of Epstein–Barr virus (EBV), is generally sufficient to initiate the lytic cycle of a gammaherpesvirus. The promoters of gammaherpesvirus transactivators are therefore silenced in latently infected cells until turned on by as yet poorly defined extracellular signals (Binne et al., 2002). The key lytic cycle transactivator in gamma-2-herpesviruses is encoded by ORF50 (Sun et al., 1998). ORF50 transcription is both necessary and sufficient to drive the entire lytic cycle of either the Kaposi’s sarcoma-associated herpesvirus (KSHV) (Gradoville et al., 2000; Lukac et al., 1999) or the related murine gammaherpesvirus-68 (MHV-68) (Wu et al., 2000, 2001). Silencing of the KSHV ORF50 promoter contributes to the maintenance of latency in vitro (Chen et al., 2001). Here we have used MHV-68 to study the role of ORF50 transcriptional control in host colonization.

MHV-68 is a natural pathogen of small rodents (Blaskovic et al., 1980), including Apodemus sylvaticus (field mice) (Blasdell et al., 2003). After intranasal injection of inbred strains of laboratory mice (Mus musculus/domesticus), MHV-68 replicates lytically in respiratory epithelial cells and then establishes life-long latency in lymphoid tissue. As with EBV and KSHV, B cells provide the principal latent reservoir of MHV-68 (Sunil-Chandra et al., 1992a). MHV-68 can also be latent in macrophages (Weck et al., 1999), epithelial cells (Stewart et al., 1998) and dendritic cells (Flano et al., 2000), and still persists in B cell-deficient mice. However, this persistence seems to reflect chronic lytic infection more than the establishment of a stable latent viral reservoir (Gangappa et al., 2002). Latency in B cells is therefore crucial to normal host colonization. Another feature MHV-68 shares with the human gammaherpesviruses is its capacity to drive B cell proliferation. There is a massive expansion of latently infected B cell numbers in lymphoid germinal centres after primary infection (Simas & Efstathiou, 1998), which probably allows MHV-68 to disseminate to sites such as the bone marrow (Sunil-Chandra et al., 1992b) and to establish a latent reservoir for life-long persistence. The latency expansion also drives an infectious mononucleosis-like illness, characterized by lymphadenopathy, splenomegaly and widespread lymphocyte activation (Doherty et al., 2001).
MHV-68 infection of conventional mice is an established model of gammaherpesvirus pathogenesis. Thus by defining the functions that contribute to MHV-68 lymphocyte proliferation and persistence in vivo, we can identify potential therapeutic targets in the clinically important gammaherpesvirus infections of humans and the economically important gammaherpesvirus infections of domesticated ungulates. We have previously used the deletion of immune evasion genes to generate MHV-68 mutants with a limited capacity for latency amplification (BridgeMAN et al., 2001; Stevenson et al., 2002). However, the equivalent immune evasion functions in other gammaherpesviruses are not always obvious, perhaps limiting the general applicability of this approach to vaccine design. Consequently, we have now used a more general strategy of attenuation, and deregulated the transcription of the MHV-68 ORF50 (LIU et al., 2000) by inserting a truncated murine cytomegalovirus (MCMV) IE1 promoter (DORSCH-HASLER et al., 1985) in its 5′-untranslated region. Our aim has been to force entry into the viral lytic cycle and to determine the effect this has on host colonization. In the accompanying paper (BONAME et al., 2004) we have explored the vaccine potential of the deregulated virus.

METHODS

Mice. Female C57BL/6 mice were purchased from Charles River UK Ltd (www.criver.com) and housed at the Biological Services Unit of the Cambridge University Department of Pathology. All procedures were carried out in accordance with Home Office Project Licence 80/1579. Mice were infected intranasally at 6–8 weeks of age with 2 × 105 PFU virus in a volume of 30 µl. To deplete T cell subsets, mice were given intraperitoneal anti-CD4 mAb (GK1.5) or anti-CD8 mAb (2.43) ascites (kindly provided by Dr P. C. Doherty, St Jude Children’s Research Hospital, Memphis, TN, USA) from 3 days before infection and every 3 days until the time of sampling.

Cell lines. Baby Hamster Kidney cells (BHK-21) (ATCC CCL-10), L929 cells (ATCC CCL-1), NIH-3T3 cells (ATCC CRL-1658), the cre-expressing derivative NIH-3T3-CRE (Stevenson et al., 2002) and murine embryonic fibroblasts (MEFs) were all grown in Dulbecco’s Modified Eagle Medium (Invitrogen) supplemented with 2 mM glutamine, 100 U penicillin ml−1, 100 µg streptomycin ml−1 and 10% fetal calf serum (PAA Laboratories) (complete medium).

Virus titres. Lungs were homogenized in complete medium, frozen, thawed and sonicated. Tissue debris was pelleted by brief centrifugation (1000 g, 1 min). Infectious virus in homogenate supernatants was measured by plaque assay on MEF monolayers as described (VIRGIN et al., 1997). The mutant BamN fragment was subcloned into the shuttle vector pST76K-SR and inserted into the MHV-68 BAC by transient ReCA-mediated recombination (ADLER et al., 2000). A revertant virus, with the unmutated BamN fragment recombined into the BAC in place of the mutant form, was then made in a similar way. A control mutant virus was made by inserting part of an MHV-68 intron upstream of ORF73 (genomic co-ordinates 105095–104879; H. M. Coleman & P.G. Stevenson, unpublished data) into the same site.

To make an ORF48-deficient virus, we cloned a Clal genomic fragment (genomic co-ordinates 62 126–69 177) into pUC19 and removed part of ORF48 (65 581–66 582) by digestion with PspMI1 (65991) and AflII (66463), in-filling with Klenow fragment DNA polymerase and religation. The mutant Clal fragment was excised, in-filled and subcloned into the Smal site of pST76K-SR, followed by ReCA-mediated recombination into the MHV-68 BAC as above. Infectious virus was reconstituted from each BAC by transfecting 5 µg BAC DNA into NIH-3T3 cells with Fugene-6 (Roche Diagnostics). The loxP-flanked BAC/GFP cassette was then removed by viral passage through NIH 3T3-CRE cells until GFP+ cells were no longer visible. The identity of each recombinant virus was confirmed by DNA sequence analysis of a PCR product spanning the mutation site. Viral stocks were grown and titrated in BHK-21 cells.

Flow cytometry. Spleens were disrupted into single-cell suspensions, washed in PBS/0.1% BSA/0.01% azide and incubated for 15 min on ice with 5% mouse serum, 5% rat serum and anti-CD16/32 mAb. Specific staining was with phycoerythrin (PE)-conjugated anti-CD8 and fluorescein isothiocyanate (FITC)-coupled anti-TCR Vβ4, anti-CD69-FITC and anti-CD19-PE, or anti-CD62L-PE (all from BD-Pharmingen), anti-CD4-FITC (Serotec), and anti-CD8-tricolor (Caltag Laboratories). After 1 h on ice, unbound antibody was removed by washing twice in PBS/BSA (0.1%)/azide (0.01%) and cells were analysed on a FACS Calibur using Cellquest software (Becton–Dickinson). Data were graphed with FCSExpress v1.3 (www.fcspress.com). For flow cytometric sorting, red cells were removed by flash lysing in water and spleen cell suspensions were stained with anti-CD19-PE and anti-I-Ab-FITC (Serotec). After washing, CD19+I-Ab− and CD19−I-Ab+ populations were separated using a FACStar plus (Becton–Dickinson).

Analysis of viral RNA. RNA was extracted from MHV-68-infected cells with RNazol-B (Tel-Test Inc.). Samples were then processed for Northern blotting or cDNA synthesis. For Northern blotting, total RNA was electrophoresed (5 µg per lane) on a 1% formaldehyde agarose gel and blotted overnight onto positively charged nylon membranes (Roche Diagnostics). Probes for β-actin, ORF48, ORF49, ORF50 and M7 were generated byPCR of either cellular cDNA or MHV-68 BAC DNA, gel-purified and random-prime-labelled (Qbiogene) with 32PdCTP (Amersham Biosciences). Blots were washed (0×2× SSC, 0.1% SDS, 65°C) and exposed to X-ray film. For cDNA synthesis, any contaminating DNA was first removed with RNase-free DNase (Promega). For the detection of ORF48 and ORF49 transcripts, cDNA was synthesized with AMV reverse transcriptase (Promega) using an oligo-dT primer and amplified with Taq polymerase (Amersham Biosciences), according to the manufacturers’ instructions. The primers used corresponded to genomic co-ordinates 66 589–66 565 and 65 581–65 604 for ORF48, and 67 660–66 639 and 66 735–66 753 for ORF49. Reactions were run for 30 cycles with an annealing temperature of 55°C. To
identify the ORF50 transcription start site we used 5′ RACE (5′/3′ RACE kit; Roche Diagnostics), according to the manufacturer’s instructions. RNA was reverse-transcribed with a primer corresponding to genomic co-ordinates 68 050–68 030, a poly-dA tail was added to the 5′ end with terminal transferase and the product was amplified with a tail-specific primer and primers corresponding to ORF50 exon 2 (genomic co-ordinates 68 026–68 005 or 67 997–67 976).

**Analysis of viral DNA.** For Southern blot analysis of viral genomes, DNA was purified from infected BHK-21 cells by Proteinase K digestion, phenol/chloroform extraction and salt/ethanol precipitation. DNA (5 μg) was restriction digested, electrophoresed, transferred onto Hybond nylon filters and hybridized to a probe prepared by random-primed [32P]dCTP labelling of the BamN genomic fragment. Filters were washed (0·2 × SSC, 0·1 % SDS, 65 °C) and exposed to X-ray film. For PCR detection of viral genomes, DNA was purified from tissue fragments using the Wizard DNA genomic purification kit (Promega). We used either Taq polymerase to amplify part of the viral M7 gene (genomic co-ordinates 69 526–69 913) over 30 cycles, or AmpliTaq Gold (Roche Diagnostics) to amplify part of ORF57 (genomic co-ordinates 75 841–76 202) over 50 cycles. The latter was sufficient for single-copy template detection. All PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining.

**In situ hybridization.** Cells expressing viral tRNAs 1–4 were detected by in situ hybridization of formalin-fixed, paraffin-embedded spleen cell sections with a digoxigenin-labelled riboprobe, transcribed from pEH14 as previously described (Bridgeman et al., 2001). Bound probe was detected with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Boehringer Ingelheim) according to the manufacturer’s instructions.

**RESULTS**

**M50 replication in vitro**

MHV-68 entry into the lytic cycle was deregulated by cloning a 416 bp fragment of the MCMV IE1 promoter into the 5′ untranslated region of ORF50. Thus the native ORF50 promoter was left intact, but ORF50 transcription could now also be initiated from a downstream site under the control of a new, more powerful promoter (Fig. 1a). The structure of the mutant virus (M50) and its revertant (50R) were checked by restriction enzyme mapping of BAC DNA (not shown) and by Southern blotting of viral DNA (Fig. 1b). RACE analysis of mRNA isolated from BHK-21 cells 6 h after infection with the M50 virus confirmed that the predominant ORF50 transcript now originated at a position corresponding to the published MCMV IE1 start site (Dorsch-Hasler et al., 1985) (Fig. 1c).

An accelerated in vitro growth phenotype of the M50 virus was immediately apparent from the rapid formation of large plaques after BAC DNA transfection into BHK-21 cells. Low multiplicity growth curves confirmed that the M50 virus replicated more rapidly than wild-type (WT) or M50R in a range of permissive cell lines (Fig. 2). A control virus, made by inserting a DNA fragment of equivalent size but without promoter activity into the same site, showed normal growth kinetics (Fig. 2). Thus even though MHV-68 infection of fibroblasts is predominantly lytic, ORF50 expression must normally limit either the rate of entry into the lytic cycle or the rate of progress through it. The accelerated growth of the M50 virus was most obvious in L929 cells (Fig. 2b), which supported WT virus spread relatively poorly. Single-cycle lytic replication in L929 cells (Fig. 2d) proceeded approximately twice as fast with the M50 virus as with WT. The M50 virus also showed increased lytic infection of N50 myeloma cells, which normally support a mainly latent infection (Sunil-Chandra et al., 1993); while N50 cell cultures infected with WT or 50R MHV-68 could be maintained long-term, those infected with M50 virus were all lysed after 2–6 weeks.

Northern blot analysis confirmed that the M50 virus transcribed ORF50 at a much higher level than did WT or 50R viruses (Fig. 3a). A greater loss of β-actin transcripts was also observed with the M50 virus, implying that host shutoff proceeded more rapidly. We quantitated transcripts from the neighbouring ORFs 48 and 49 by Northern blotting of BHK-21 cells after overnight infection (Fig. 3b). Neither was increased, arguing that the MCMV IE1 promoter fragment specifically up-regulated ORF50 transcription. The small reductions in ORF48 and ORF49 mRNA with the M50 virus (Fig. 3b) were consistent with its faster progression through the lytic cycle. These transcripts peak relatively early during MHV-68 infection (Ahn et al., 2002), so by 18 h after infection, the M50 virus had probably progressed further beyond maximal ORF48 and ORF49 expression than had WT or 50R. At 6 h after infection, non-saturating RT-PCR analysis showed that ORF48 and ORF49 transcripts were at least as abundant with the M50 virus as with WT (Fig. 3c). Allowing for the altered kinetics of M50 virus lytic cycle gene expression, therefore, the transcription of the ORFs neighbouring the mutation site appeared to be intact. The fact that a control insertion into the same site as the MCMV IE1 promoter had no effect on the rate of MHV-68 lytic replication (Fig. 2) further argued against the disruption of a viral function unrelated to ORF50 transcription being responsible for the M50 phenotype.

**M50 replication in vivo**

The M50 virus caused little or no sign of clinical illness after intranasal infection of C57BL/6 mice. Infectious centre assays showed that low levels of virus seeded to the spleen, but then failed to be amplified (Fig. 4a). While WT and 50R titres increased, M50 infectious centre titres declined after day 7 of infection, implying that latency could not be maintained. By 13 days after infection, when the WT and 50R viruses had reached peak latency, replication-competent M50 virus could not be recovered. Infectious centres remained undetectable with the M50 virus at 6 months after infection (data not shown). The control insertion mutant showed no latency defect (Fig. 4a), arguing again that the M50 phenotype was not due to a disruption of viral functions unrelated to ORF50. Since the proximity of the 5′ end of ORF48 to the M50 insertion site meant that this was the gene most likely to be accidentally
compromised, we further tested latency establishment by a targeted ORF48 mutant (Fig. 4a). This virus showed no defect in splenic latency establishment.

In addition to its latency deficit, the M50 virus was cleared somewhat more rapidly from the respiratory tract than WT or 50R controls, although this reduction in lytic virus was relatively minor compared to the severe reduction in latency. Infectious M50 titres were equivalent to those of WT and 50R after 3 days of infection, but then showed a relative decline, with clearance to undetectable levels by 10 days. The control insertion mutant showed no such deficit (Fig. 4a).

The M50 virus recovered from the spleen at days 5 and 7 of infection (Fig. 4A) was apparently latent, since no pre-formed infectious virus was detected in freeze-thawed spleen cells. However, extrapolating a single-cell definition of latency to a whole organ – virus recoverable from live but not from killed cells – is problematic with low levels of virus. For example, a rapid uptake of new virions by uninfected spleen cells – a short half-life of infectious

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**Fig. 1.** A hybrid promoter for the MHV-68 ORF50. (a) The genomic locations of ORF50 and the MCMV IE1 promoter insertion site are shown. The MHV-68 ORF50 promoter overlaps the 5’ end of ORF48, and transcription starts between ORF48 and ORF49 (Liu et al., 2000). A short exon 1 is spliced over ORF49 onto exon 2. (b) BamHI- (Bam), HindIII- (Hin) and BglII-digested DNA from virus-infected cells was electrophoresed, transferred to nylon membranes and probed with a BamHI genomic clone, as indicated in (a). (c) RNA isolated from BHK-21 cells 6 h after infection with the M50 virus was reverse-transcribed using an ORF50-specific primer (genomic co-ordinates 68,050–68,030). A 5’ poly-dT tail was added with terminal transferase and cDNA was amplified using a tail-specific primer and one corresponding to genomic co-ordinates 67,997–67,976 (left-hand lane) or 68,026–68,005 (right-hand lane). The complementary strand sequence obtained from the predominant PCR product (400–500 bp) in each case is shown up to the added 5’ poly-A tail. The MCMV IE1 promoter sequence is shown in italic type and the MHV-68 sequence in regular type, joined at a BsgI restriction digestion site. The ORF50 start codon is in bold type and the exon 1/exon 2 splice junction (GG) is in bold italic type.
particles rather than latency – could also have accounted for the absence of pre-formed infectious virus. In situ hybridization of mediastinal lymph nodes for viral tRNA expression 7 days after infection showed no evidence of the M50 infection compared to WT and M50R controls (Fig. 5). Thus, by this marker of latency (Bowden et al., 1997), M50 lymphoid colonization was below detectable limits even at its peak.

Because ORF50 initiates the viral lytic cycle, it must be made before lytic cycle immune evasion becomes operative. A possible explanation of the in vivo deficit in M50 virus replication was therefore that up-regulating ORF50 expression made it an immune target. For example, human cytomegalovirus relies on pp65 to limit CD8$^+$ T cell epitope presentation from its IE1 transactivator (Gilbert et al., 1996); such a mechanism might be overwhelmed by higher levels of transcription. Compromised immune evasion would predict some reversal of the M50 deficit by immune depletion, in the same way that CD8$^+$ T cell depletion largely reverses the MHV-68 latency establishment deficit associated with M3 disruption (Bridgeman et al., 2001) and completely reverses the deficit associated with K3 disruption (Stevenson et al., 2002). However, M50 virus titres remained low in mice depleted of CD4$^+$ or CD8$^+$ T cells (Fig. 4b). [The low infectious centre titres of all viruses in CD4$^+$ T cell-depleted mice corresponds to the CD4$^+$ T cell dependence of MHV-68 latency amplification (Usherwood et al., 1996.)] The small increase in M50 infectious centre titres in the spleen with T cell depletion, most obvious with CD8$^+$ T cell depletion, was difficult to interpret because of some increase also in infectious virus (day 12, Fig. 4b). As the deficit in M50 infectious centres relative to WT and 50R remained substantial, there was little evidence of ORF50 overexpression making MHV-68 more susceptible to either T cell subset.

In addition to achieving lower infectious centre titres, the M50 virus caused little of the splenomegaly (not shown) or virus-driven T cell and B cell activation and CD8$^+$Vβ4$^+$ T cell expansion (Fig. 6) that normally characterize the MHV-68-associated infectious mononucleosis illness. Since it is the amplification of latent virus that drives this host immune activation (Doherty et al., 2001), the relative lack of immune activation was further evidence that the M50 virus was unable to maintain normal latency.

Reduced M50 viral DNA load

We also sought to confirm compromised M50 latency establishment by measuring viral DNA loads. DNA was extracted from spleens 18 days after infection with M50, WT or 50R viruses and a portion of the MHV-68 M7 ORF was amplified by PCR (Fig. 7a). This showed a clear reduction in the M50 viral DNA load (Fig. 7a). For a more quantitative comparison, we used PCR with single-copy sensitivity to amplify DNA corresponding to part of ORF57 from limiting amounts of template DNA (Fig. 7b). This showed that DNA loads with the M50 virus were at least 10- to 100-fold lower than with WT or 50R viruses.
We also purified MHC class II^+CD19^+ (B cell) and MHC class II^+CD19^- (macrophage and dendritic cell) populations by flow cytometric sorting and compared their viral DNA loads (Fig. 7c). WT virus showed a similar viral DNA load in CD19^+ and CD19^- populations, whereas the M50 virus showed a relative lack of viral DNA in CD19^- B cells. This was consistent with a failure of the M50 virus to drive latency amplification, which is associated with the infection of germinal centre B cells (Flano et al., 2002; Marques et al., 2003).

DISCUSSION

Latency is a cardinal feature of all herpesviruses. We disrupted MHV-68 latency by introducing a strong promoter element upstream of the gene encoding the MHV-68 major lytic transactivator, ORF50. Despite enhanced replication of the mutant virus in vitro, it showed severely impaired host colonization in vivo with a principal defect in latency establishment. The M50 virus was consequently well-controlled even in immunocompromised mice. These results underlined the crucial importance that lytic cycle transcriptional silencing plays in gammaherpesvirus host colonization.

Even though MHV-68 fairly readily enters the lytic cycle in in vitro cell lines, it was evident that ORF50 availability is still a limiting factor. Clearly a similar modification of the EBV BZLF-1 or KSHV ORF50 promoters may be one means of generating an in vitro lytic replication system for these viruses as well. Up-regulating ORF50 production presumably increased the probability of initiating lytic infection rather than speeding up the lytic cycle itself, which is likely to be limited by substrate supply for virion assembly. The fact that up-regulating ORF50 transcription reduced rather than increased lytic spread in vivo argued that here exogenous host factors are the dominant influence on virus replication, even before the onset of adaptive immunity (Fig. 4a, days 3–5). The interferon system is one likely candidate (Dutia et al., 1999).

The insertion of self-contained expression cassettes into the left end of the MHV-68 genome can cause latency-associated virus attenuation (Jacoby et al., 2002; Adler et al., 2001), perhaps as a consequence of driving the high-level expression of reporter genes (β-galactosidase or green fluorescent protein) in latently infected cells. However, two viruses with left-end lacZ expression cassettes have established normal latency (Simas et al., 1998) and a green fluorescent protein expression cassette inserted into the MHV-68 K3 ORF caused no more attenuation than a small non-coding insertion in the same site (Stevenson et al., 2002). Thus another possible explanation for the attenuating effect of certain insertions is that they disrupt specific viral functions at the left end of the genome. Even this attenuation was much less severe than seen with the M50 virus – a latency reduction of one rather than three or four orders of magnitude. Thus it seemed unlikely that the MCMV IE1 promoter insertion caused attenuation by a non-specific effect of increased transcription.

The incapacity of the M50 virus to amplify latently infected B cell numbers was presumably due to the MCMV IE1

Fig. 3. Transcript levels after viral infection of BHK-21 cells. (a) Northern blot of RNA extracted from cells either uninfected (UI) or 6 h after infection with WT, M50 or 50R virus. Replicate blots were probed for ORF50 or β-actin. (b) Northern blots of RNA after overnight infection with the virus indicated, as in (a). Replicate filters were probed for ORF48, ORF49 or M7. (c) RT-PCR analysis of ORF48 and ORF49 transcripts 6 h after infection with the virus indicated. Ethidium bromide-stained PCR products with (RT+) and without (RT-) reverse transcription are shown. The positive control (+) was amplified from viral DNA.
promoter maintaining ORF50 transcription and hence continually driving lytic virus replication. This would be consistent with the increased ORF50 transcription and lytic replication observed with the M50 virus in vitro. We were not able to define directly the in vivo behaviour of the M50 MCMV IE1 promoter fragment because so few infected lymphoid cells were ever found. However, the selective reduction of viral DNA in B cells and fact that the M50 virus inevitably lysed NS0 cells supported the idea of destabilized B cell latency.

**Fig. 4.** Growth in vivo of MHV-68 with deregulated ORF50 expression. (a) Mice were infected intranasally with WT (open squares), M50 (open diamonds) or 50R (open circles) viruses. At critical time points we also titrated samples from mice infected with a virus carrying a control insertion in the 5’ untranslated region of ORF50 (filled circles). At 23 days after infection, infectious centre titres were compared between wild-type (open squares), M50 (open diamonds) and ORF48-deficient viruses (filled squares). Mean ± SEM titres of five mice per time point. (b) Mice were depleted of CD4+ (CD4“) or CD8+ (CD8“) T cells from 3 days before infection until the time of sampling, or were left undepleted (Nil). Infectious virus was measured by plaque assay of lung homogenates, and latent virus by infectious centre assay of spleen cells. Infectious virus in the spleen was taken as the titre present in freeze-thawed samples. Mean ± SEM titres of five mice per group are shown, infected with WT (open bar), M50 (stippled bar) or 50R (solid bar) viruses. The dotted line shows the lower limit of assay detection.
**Fig. 5.** *In situ* hybridization for viral tRNA transcripts. Mediastinal lymph nodes were removed 7 days after intranasal infection with the virus indicated and fixed in 4% formaldehyde. Paraffin-embedded tissue sections were hybridized with a DIG-labelled anti-sense riboprobe spanning viral tRNAs 1–4. Detection was with alkaline phosphatase-conjugated anti-digoxigenin Ig-Fab fragments. Arrows indicate examples of viral tRNA+ cells.

**Fig. 6.** Immune activation after infection with WT, M50 and 50R viruses. (a) T cell activation assessed by flow cytometry of CD62L down-regulation on splenic CD4+ (open bar) and CD8+ (solid bar) T cells 12 days after infection. Mean ± SD titres of five mice per group are shown. The level of CD4+ and CD8+ T cell activation with the M50 virus was significantly reduced compared to WT (P < 0.0001 by *t* test). (b) CD8+Vb4+ T cell expansion, measured by flow cytometry of spleen cells 18 days after infection. Each point represents one mouse. (c) Flow cytometric assay of the CD69 activation marker on splenic CD19+ B cells 12 days after infection. Mean ± SD titres of five mice per group are shown. Where indicated, mice were depleted of CD4+ or CD8+ T cells from just before infection until the time of sampling, as in Fig. 4(b). The level of B cell activation with the M50 virus remained significantly reduced after CD8+ T cell depletion compared to WT (P < 0.001 by *t* test).
The presence of low levels of M50 viral DNA in spleens (Fig. 7b) raised the possibility that the M50 virus was perhaps still able to establish some kind of latency, perhaps in myeloid cells (Fig. 7c). However, if so it is difficult to see why it did not reanimate in vitro after day 10 of infection. WT MHV-68 reactivates relatively efficiently from macrophages and dendritic cells (Marques et al., 2003), and transcription from the native ORF50 promoter should not have been compromised in the M50 virus: the MCMV IE1 promoter element would simply have extended the mRNA 5’ untranslated region. An alternative explanation for the viral DNA detected at low levels by PCR is that this was non-functional genomic debris, left over after the immune clearance of infected cells. Regardless of whether the M50 virus persisted at all in a viable form, the key point is that it failed to drive in vitro B cell proliferation, the pathological process usually associated with gammaherpesvirus-associated tumours. Thus we could define with MHV-68 the role of a fundamental gammaherpesvirus function – lytic cycle repression – in pathogenesis, and so demonstrate a possible general means of gammaherpesvirus attenuation. In the accompanying paper (Boname et al., 2004) we report the capacity of infection with the M50 virus to protect against a subsequent WT MHV-68 challenge.

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Fig. 7. Detection of viral DNA after infection with WT, M50 and 50R viruses. (a) A fragment of the MHV-68 M7 was amplified by PCR from 100 ng splenic DNA harvested 18 days after infection. Controls show the amplification of 10 ng splicen DNA from WT only control; +, PCR of a genomic clone spanning M7. (b) A fragment of the MHV-68 ORF57 was amplified from splenic DNA harvested 18 days after infection. --, Water only control; +, PCR of 50 ng splenic DNA from a naive mouse spiked with 10 copies of WT MHV-68 BAC DNA. Ethidium bromide-stained PCR products amplified from 50, 5 or 0.5 ng splenic DNA of individual mice are shown. (c) Spleen cells from groups of three C57BL/6 mice infected with either WT or M50 virus were harvested after 18 days, pooled and stained for cell-surface CD19 and MHC class II (I-A2A). Cells were sorted into CD19+MHC class II+ and CD19+MHC class II+ populations. DNA was then extracted and 50–0.005 ng samples were subjected to PCR amplification with ORF57-specific primers as in (b).


