The large, worldwide burden of disease linked to gammaherpesvirus infections has generated considerable interest in trying to control the extent of these viruses through vaccination. Immunization with recombinant gp340 protects against Epstein–Barr virus (EBV)-induced tumours in cotton-top tamarins (Epstein et al., 1985). However, whether this strategy will also protect the natural host of the virus against disease remains uncertain. The strict species specificity of most primate gammaherpesviruses has made it difficult to test different vaccination strategies without large, expensive and prolonged clinical trials. Consequently, few general principles have been established that might predict the efficacy of a gammaherpesvirus vaccine in limiting wild-type (WT) infection.

It is possible with the murine gammaherpesvirus-68 (MHV-68) to test the capacity of different vaccination strategies to protect against a gammaherpesvirus infecting what is probably its natural host (Blasdell et al., 2003). Subunit vaccines – including gp150, the MHV-68 homologue of the EBV gp340 (Stewart et al., 1996) – have so far proved ineffective in controlling MHV-68 latency establishment (Stewart et al., 1999; Stevenson et al., 1999a; Liu et al., 1999; Usherwood et al., 2001). The multiple immune evasion strategies of MHV-68 (Stevenson et al., 2002a) probably render ineffective the limited immunity that is generated against isolated viral components. Since an empirically attenuated strain of varicella-zoster virus (Asano & Takahashi, 1977) is the only herpesvirus vaccine to date that has proved clearly effective in preventing disease in the natural host (Wills et al., 2002), attenuated MHV-68 mutants may elicit more effective immune protection than individual viral subunits. The existence of multiple co-existing EBV strains in some individuals (Brooks et al., 2000; Sitki-Green et al., 2003, Walling et al., 2003) suggests that sterilizing immunity to gammaherpesvirus infections is probably not a realistic goal. However, it may be possible to reduce the long-term level of latency and so prevent disease.

Because it is possible with MHV-68 to identify the contribution of individual gammaherpesvirus genetic loci to pathogenesis and host colonization, we can improve on empirical strategies of attenuation and employ specific, testable, molecular-based strategies to design candidate vaccines. In the accompanying paper (May et al., 2004) we have described the generation and characterization of a latency-deficient mutant of MHV-68, M50, made by deregulating the transcription of the viral ORF50 lytic transactivator. We targeted viral latency because this makes a major contribution to MHV-68 host colonization (Coleman et al., 2003) and because latency-associated lymphocyte proliferation probably underlies most clinically important, gammaherpesvirus-associated disease. A major immediate-early transactivator that initiates the viral lytic cycle is common to all gammaherpesviruses, even though the transactivators themselves are relatively varied (Sun et al., 1998; Wu et al., 2000; Binne et al., 2002). The MHV-68 ORF50 is homologous to the ORF50 of other gamma-2-herpesviruses, including the Kaposi’s sarcoma-associated herpesvirus (KSHV) and to the Rta transactivator of EBV. Thus the strategy of MHV-68 attenuation by deregulation of ORF50 could be readily adapted to other gammaherpesviruses that cause clinically and economically important disease. Here we have tested the capacity of infection with the M50 mutant to protect against host colonization following a subsequent WT virus challenge. The aim was to provide a proof of principle that...
the immunity to a latency-deficient mutant can protect against the subsequent establishment of normal latency by WT virus.

We first measured the immune response to M50 infection (Fig. 1). The virus-specific CD4+ T cell (ELISpots with virus-infected targets), CD8+ T cell (ELISpots with epitope-pulsed targets) and B cell (serum antibody) responses were all reduced compared to those elicited with WT or revertant (50R) viruses. The IFN-γ-producing CD4+ T cell response was the most obviously affected, while the differences in virus-specific serum antibody titres were relatively small. The reduced immunogenicity of the M50 virus presumably reflected both its reduced lytic replication in the lung and its failure to achieve latency amplification (see May et al., 2004). Lytic recrudescence from the pool of genome-positive cells generated by latency amplification provides a surprisingly large load of immunogenic MHV-68 lytic antigens (Stevenson et al., 1999c, 2002b; Coleman et al., 2003), so we would expect most latency-deficient viruses to be less immunogenic than WT. However, despite its reduced immunogenicity, the M50 virus still offered significant theoretical advantages over subunit vaccines, due to the range of antigens presented and the appropriate context of their presentation. We chose to retain the M3 and MK3 immune evasion genes in the M50 vaccine virus again to provide appropriate levels of antigen in an appropriate infectious context. MK3-deficient MHV-68 elicits higher frequencies of virus-specific CD8+ T cells than WT virus (Stevenson et al., 2002b), but the capacity of these cells to control a virus with intact immune evasion remains unproven; CD8+ T cells stimulated by high levels of MHC class I/peptide complexes are not always effective when the numbers of these complexes on infected target cells are small.

With any attenuated virus vaccine there clearly has to be a trade-off between pathogenicity and immunogenicity. Completely replication-deficient virus vaccines tend to offer little long-term immunity, particularly if they do not persist. The immunity elicited by heat-inactivated MHV-68 thus affords little protection against a subsequent challenge with live virus (Arico et al., 2002). Conversely, a gammaherpesvirus that still establishes normal latency, while it affords some protection against a subsequent, low-dose WT infection (Tibbetts et al., 2003) is itself unlikely to be safe. The aim of vaccination has to be to minimize virus-induced lymphocyte proliferation and reduce the long-term latent viral load. The M50 mutation sacrificed some immunogenicity in order to minimize vaccine-associated lymphocyte proliferation. With current technology, some such compromise is likely to be necessary in disease control.

To test the protection afforded by M50-specific immunity, we vaccinated mice intranasally with M50 virus

![Fig. 1. Immune response to the M50 virus. (a) Spleen cells were harvested 18 days after intranasal infection of C57BL/6 mice and assayed for virus-specific CD4+ and CD8+ T cells by IFNγ ELISpot as described previously (Stevenson et al., 1999b). Stimulating target cells were irradiated splenocytes from syngeneic, naïve mice, either untreated (nil), exposed to WT MHV-68 (2 p.f.u. per cell) to detect CD4+ T cell responses (virus) (Christensen & Doherty, 1999), or pulsed with 1 µM AGPHNDEMI (p56) or TSINFVKI (p79) peptides to detect CD8+ T cell responses. Secreted IFNγ was captured on nitrocellulose membranes (Millipore) with mAb R4-6A2 (BD-Pharmingen). After 48 h culture at 37 °C in RPMI supplemented with 2 mM glutamine, 100 U penicillin ml−1, 100 µg streptomycin ml−1, 10% fetal calf serum (PAA Laboratories) and 20 U human recombinant IL-2 ml−1, captured IFNγ was detected with biotinylated mAb XMG1.2 (BD-Pharmingen), streptavidin-alkaline phosphatase (Dako) and BCP/NBT substrate (Roche Diagnostics). Mean ± SD responses of five mice per group are shown. (b) Virus-specific and total serum IgG levels in C57BL/6 mice infected 18 days earlier were measured by ELISA. Maxisorp ELISA plates (Nalge Nunc) were coated overnight with either affinity-purified goat anti-mouse IgG sera (Sigma) or 0-05% Triton X-100-disrupted MHV-68 as described previously (Stevenson & Doherty, 1999). After incubation with threefold serum dilutions, bound murine IgG was detected with alkaline phosphatase-conjugated goat anti-mouse IgG-Fcγ sera and nitrophenylphosphate substrate (Sigma). Serum IgG titres were calculated by comparison to a standard immune serum included on each plate, so the values are relative rather than absolute. Mean ± SD titres of five mice per group are shown.](image-url)
(2 × 10⁴ p.f.u.) and 3 months later infected these mice and age-matched, unvaccinated controls intranasally with WT virus (2 × 10⁴ p.f.u.). There was an almost complete ablation of lytic virus replication in the lungs and latent viral amplification in the spleens of the vaccinated mice (Fig. 2). There was also no evidence in the vaccinated mice of the latency-associated splenomegaly or CD8⁺Vβ4⁺ T cell expansion normally driven by WT virus. Thus even the limited immunity afforded by M50 infection was sufficient to control WT viral lytic replication and latency amplification.

We have previously observed delayed latency establishment by WT virus when lytic replication is inhibited by prior vaccination (Stevenson *et al*., 1999a). An almost complete absence of lytic replication appears to delay the establishment of latency by 10–20 days. Thus we looked for delayed latent colonization of M50-vaccinated mice with WT virus by PCR-based detection of viral DNA 3 months after challenge (Fig. 3). To distinguish WT from M50 viral DNA, we used PCR primers that amplified across the MCMV IE1 promoter insertion site in the M50 virus. This gave a 239 bp product with WT viral DNA and a 655 bp product with M50 viral DNA. PCR products were transferred to nylon membranes and hybridized with [³²P]dCTP-labelled BamHI genomic clone (co-ordinates 64765–68813) to ensure detection specificity and increase detection sensitivity.

Control reactions established that there was single-copy sensitivity detection of WT viral DNA (Fig. 3c). M50 viral DNA was detected with approximately 10-copy sensitivity (not shown). Template mixing controls (Fig. 3c) established that the presence of M50 viral DNA did not reduce the detection of WT viral DNA, even when present in 10-fold excess. WT viral DNA was detected in all spleens (Fig. 3a) and all lungs (Fig. 3b) of unvaccinated mice. Tenfold DNA dilutions established that spleens contained approximately one copy of viral DNA per 1 ng total DNA (Fig. 3c). Spleens of vaccinated mice contained approximately one copy of WT viral DNA per 1000 ng total DNA (Fig. 3a). Thus vaccination reduced the level of WT viral persistence 1000-fold. Although there was > 10-fold variation in WT viral DNA in lungs between individual vaccinated mice, it was clear that vaccination achieved a similar reduction in this site. M50 viral DNA was undetectable in all vaccinated mice.

Disease prevention relevant to EBV or KSHV is difficult to assess directly in the MHV-68 model because the murine virus, despite one report (Sunil-Chandra *et al*., 1994), is not obviously tumorigenic in standard mouse strains, even with immune suppression. Thus a direct correlate of the desirable outcome of an EBV or KSHV vaccine – preventing tumours – is currently not possible. However, despite not being tumorigenic, MHV-68 does drive lymphocyte proliferation. This results in a massive increase in latently infected B cell numbers in the infectious-mononucleosis-like illness, with widespread lymphocyte activation and a marked expansion in the CD8⁺Vβ4⁺ T cell subset (Doherty *et al*., 1997). Thus a reduction in MHV-68 latency establishment and MHV-68-driven mononucleosis probably reflects a reduction in virus-driven lymphocyte proliferation, the process that underlies gammaherpesvirus-associated tumours.

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**Fig. 2.** Replication of WT MHV-68 after intranasal infection of vaccinated mice. C57BL/6J mice were infected intranasally with the M50 mutant (2 × 10⁴ p.f.u.) (open circles) or were left uninfected (filled circles). Three months later, all mice were infected intranasally with WT virus (2 × 10⁴ p.f.u.).

(a) Infectious virus in lung homogenates was measured by plaque assay. (b) Latent virus in spleens was measured by infectious centre assay using murine embryonic fibroblast monolayers as described previously (Coleman *et al*., 2003). (c) Spleen weights were used as an indicator of virus-driven splenomegaly. (d) Expansion of the CD8⁺Vβ4⁺ T cell subset was assayed by flow cytometric staining of splenocytes as described previously (Coleman *et al*., 2003). Each point represents an individual mouse.
M50-vaccinated mice did show a clear inhibition of latency establishment by superinfecting WT virus.

The fact that the M50 virus itself is impaired in latency amplification meant that the total latent viral load in vaccinated mice remained low. It is possible that M50 virus might accumulate compensatory mutations to prevent entry into the lytic cycle and so maintain latency. However, the primed immune response should limit the size of an M50 latent pool in the same way that it limited the WT latent load. It seems unlikely that vaccination with the M50 virus can completely prevent WT infection – each PCR reaction, after all, samples only a small fraction of the whole organ – but it did appear possible to limit WT virus-driven lymphocyte proliferation without this being a characteristic of the immunizing virus. It should presumably be possible to employ a similar strategy with other gammaherpesviruses so as to protect against the disease associated with WT viral latency.

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References


