Comparative study of murid gammaherpesvirus 4 infection in mice and in a natural host, bank voles

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Received 11 May 2010
Accepted 8 June 2010

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Gammaherpesviruses are archetypal pathogenic persistent viruses. The known human gammaherpesviruses (Epstein–Barr virus and Kaposi’s sarcoma-associated herpesvirus) are host-specific and therefore lack a convenient in vivo infection model. This makes related animal gammaherpesviruses an important source of information. Infection by murid herpesvirus 4 (MuHV-4), a virus originally isolated from bank voles (Myodes glareolus), was studied here. MuHV-4 infection of inbred laboratory mouse strains (Mus musculus) is commonly used as a general model of gammaherpesvirus pathogenesis. However, MuHV-4 has not been isolated from house mice, and no systematic comparison has been made between experimental MuHV-4 infections of mice and bank voles. This study therefore characterized MuHV-4 (strain MHV-68) infection of inbred laboratory mouse strains (Mus musculus) is commonly used as a general model of gammaherpesvirus pathogenesis. However, MuHV-4 has not been isolated from house mice, and no systematic comparison has been made between experimental MuHV-4 infections of mice and bank voles. This study therefore characterized MuHV-4 (strain MHV-68) infection of bank voles through global luciferase imaging and classical virological methods. As in mice, intranasal virus inoculation led to productive replication in bank vole lungs, accompanied by massive cellular infiltrates. However, the extent of lytic virus replication was approximately 1000-fold lower in bank voles than in mice. Peak latency titres in lymphoid tissue were also lower, although latency was still established. Finally, virus transmission was tested between animals maintained in captivity. However, as observed in mice, MuHV-4 was not transmitted between voles under these conditions. In conclusion, this study revealed that, despite quantitative differences, replication and the latency sites of MuHV-4 are comparable in bank voles and mice. Therefore, it appears that, so far, Mus musculus represents a suitable host for studying gammaherpesvirus pathogenesis with MuHV-4. Establishing transmission conditions in captivity will be a vital step for further research in this field.

INTRODUCTION

Gammaherpesviruses have been identified in a range of animals from mice to man (Davison et al., 2009). They establish persistent, productive infections, with virus carriers making antiviral immune responses that protect against disease and continuing to secrete infectious virions. Most gammaherpesviruses establish a long-term latent infection of circulating lymphocytes. They drive lymphocyte proliferation as part of normal host colonization, and this feature of the virus life cycle predisposes the host to neoplastic disease. Such disease can be particularly marked when cross-species transmission occurs, as observed for saimiriine herpesvirus 2, ovine herpesvirus 2 and alcelaphine herpesvirus 1 (Dewals et al., 2006; Fickenscher & Fleckenstein, 2001; Hart et al., 2007).

The best studied gammaherpesviruses are human herpesvirus 4 (Epstein–Barr virus) and human herpesvirus 8 (Kaposi’s sarcoma-associated herpesvirus), which are associated with a range of cancers. As these viruses have no well-established in vivo infection model, related animal gammaherpesviruses are an important source of information. We have been studying murid herpesvirus 4 (MuHV-4). The archetypal MHV-68 strain was originally isolated from bank voles (Myodes glareolus) in Slovakia (Blaskovic et al., 1980) together with four other MuHV-4 strains,

A supplementary figure showing IVIS Spectrum sensitivity in Myodes glareolus is available with the online version of this paper.
MHV-60 and -72 also isolated from bank voles and MHV-76 and -78 isolated from yellow-necked mice (Apodemus flavicollis). More recently, closely related viruses have been isolated from shrew (Crocidura russula) (Chastel et al., 1994) and wood mouse (Apodemus sylvaticus) (Blasdell et al., 2003; Hughes et al., 2010b).

Although MuHV-4 has not been isolated from house mice (Mus musculus), infection of inbred laboratory mouse strains is commonly accepted as a viable model for studying gammaherpesvirus pathogenesis in vivo. Experimental MuHV-4 infection typically employs intranasal virus inoculation under general anaesthesia. This leads to a lytic infection of lung alveolar epithelial cells that is controlled within 2 weeks (Sunil-Chandra et al., 1992). Meanwhile, the virus seeds to lymphoid tissue, mainly draining lymph nodes and spleen (Milho et al., 2009), and drives the proliferation of latently infected B cells. This peaks at 2 weeks post-infection (p.i.) and is controlled by 4 weeks. A predominantly latent infection of memory B cells then persists (Flano et al., 2002). Some inbred mouse strains infected with MuHV-4 tend to develop lymphomas (Sunil-Chandra et al., 1994).

An unresolved feature of the MuHV-4/Mus musculus infection model is that virus re-excretion and transmission have not been observed. Whilst this could mainly reflect the restrictions on normal murine social behaviour imposed by conventional housing, it is also possible that the lack of transmission reflects the fact that one or more virus functions necessary for efficient host exit work(s) poorly in carrier mice. The different host species infectable by MuHV-4-like viruses are indeed separated by several millions of years (Fig. 1). In this study, we therefore characterized MuHV-4 infection in bank voles, the species from which it was first isolated, in order to reveal any major defects of the Mus musculus infection model.

RESULTS

Luciferase imaging sensitivity in bank voles

The purpose of this study was to compare MuHV-4 infection in mice, either inbred or outbred, and in one reported natural host, the bank vole. We first monitored infection by bioluminescence imaging of animals infected with luciferase-expressing MuHV-4 (Milho et al., 2009). As bank voles have darker fur pigmentation than the BALB/c or CD1 mice used for comparison, we first established the sensitivity of bioluminescence imaging for each host. Different numbers of baby hamster kidney (BHK-21) cells infected with a MuHV-4 strain expressing luciferase under the control of the M3 promoter were therefore injected subcutaneously on the ventral part of animals before imaging. Removing the fur from animals prior to imaging had little effect on the signal obtained (data not shown), so all animals were imaged with fur present. After bioluminescent imaging, the total flux of photons for each injection site was reported on a graph (see Supplementary Fig. S1, available in JGV Online). Each group showed a similar sensitivity and linearity of signal with injected BHK-21 cell number. Therefore, bioluminescence imaging was considered a viable means of comparing infections in the different hosts.

Luciferase imaging of MuHV-4 infection in bank voles and BALB/c and CD1 mice

We then infected anaesthetized animals intranasally with 10⁵ p.f.u. luciferase+ MuHV-4 and tracked infection by d-luciferin injection and charge-coupled-device (CCD) camera scanning. Based on previous analysis (Milho et al., 2009), we considered thoracic signals to come from the lungs, abdominal signals from the spleen and neck signals from the superficial cervical lymph nodes (SCLNs). The signal intensities in the nose (Fig. 2a), lungs (Fig. 2b), SCLNs (Fig. 2c) and spleens (Fig. 2d) were monitored over time. A strong signal was visible in the lungs and noses of BALB/c mice at the peak of lytic replication (5–7 days p.i.), CD1 mice were very similar. In contrast, bank voles showed no signal in the nose and only sporadic weak signals in the lungs. At the peak of latency amplification (12–15 days p.i.), luciferase signals were weak or undetectable in the lungs and noses of most of BALB/c and CD1 mice, but strong in the SCLNs and spleen. Again, the corresponding bank vole signals were weak or undetectable.

These results were confirmed by ex vivo imaging of dissected organs (Fig. 3). In the noses of all groups, we observed sporadic signals at day 7 p.i., which had

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**Fig. 1.** Evolutionary relationships among the studied species. Synthetic phylogenetic tree summarizing the evolutionary relationships and an estimation of the divergence times among the studied species (Michaux et al., 2001, 2002; Murphy et al., 2001). Myrs, Million years.
disappeared by days 14 and 21 p.i. Signals from lungs were maximal at day 7 p.i. and again much weaker in bank voles than in mice, consistent with the analysis of living animals. Only sporadic lung signals were observed at days 14 and 21. Signals from SCLNs and spleens were maximal in mice at day 14 and only rarely observed in dissected bank voles. Based on the images, the signal in CD1 mice spleen appeared to be larger than in BALB/c mice spleen. Fig. 2 also suggested that there was more replication around day 14 in CD1 mice spleens and SCLNs than in similar organs from BALB/c mice. This probably reflects differences between mice strains and requires more experiments for clarification. No signals were observed in other organs. Therefore, it appeared that MuHV-4 follows a similar in vivo cycle in bank voles as in mice, but replicates much less well.

**Classical analysis of MuHV-4 infection in bank voles and BALB/c and CD1 mice**

Luciferase expression by luciferase+ MuHV-4 reflects predominantly lytic gene expression (Milho et al., 2009). Therefore, to explore further the establishment of MuHV-4 latency in bank voles, we infected bank voles or mice intranasally with $10^4$ p.f.u. wild-type MuHV-4 (strain MHV-68). Consistent with the bioluminescence imaging results, lytic replication, as measured by plaque assay, was greatly reduced in bank voles compared with mice (Fig. 4). At day 7 p.i., infectious virus was recovered only sporadically from noses (Fig. 4a) but was recovered from all lungs (Fig. 4b). However, plaque assay titres were approximately 1000 times lower in bank voles than in mice. Although the lung virus titres of bank voles were significantly lower than those of BALB/c mice ($P<0.001$), they were not significantly lower than those of outbred CD1 mice ($P>0.05$).

The colonization of SCLNs and spleens by latent virus was then determined by infectious centre assay. The recovery of replication-competent virus from SCLNs and spleens of mice was maximal at 14 days p.i. (Fig. 4c, d), consistent with published data (Milho et al., 2009). Virus recovery from the SCLNs and spleens of bank voles was much less consistent. However, it was possible to observe a peak latent load in spleens at 14 days p.i., suggesting that latency amplification occurs in bank voles much as it does in mice. Lower virus recovery in infection centre assays could have reflected a lower capacity of MuHV-4 to reactivate productively from latently infected bank vole spleen cells.
explanted onto BHK-21 monolayers. In order to evaluate latent loads in SCLNs and spleens further, we quantified viral genomes by real-time PCR. In spleens, the viral DNA loads of infected bank voles were approximately ten times lower than those of BALB/c mice (Fig. 5a). However, the difference was less when compared with CD1 mice. Moreover, viral DNA was recovered from the spleens of all infected bank voles, implying that MuHV-4 robustly establishes latency in this site. The SCLN latent loads were similar between bank voles and mice (Fig. 5b). Together, these results showed that MuHV-4 can efficiently establish latency in bank voles, despite lower levels of replication than in mice.

**Tissue histology and immunohistochemistry**

Dramatic peribronchiolar, perivascular and interstitial lymphocytic infiltrates were observed in mouse lungs at day 7 p.i. (Fig. 6a). These lesions were associated with virus replication as identified by immunohistochemical staining for viral antigens (Fig. 6a). A very similar interstitial pneumonia occurred in MuHV-4-infected bank voles. Viral antigens were detectable, but with a much more limited distribution than observed in mice. This was consistent with the limited luciferase expression and low virus titres of infected bank voles. Therefore, infection of bank vole lungs appeared to be qualitatively similar to that of mice, but quantitatively less.

At 14 days p.i., MuHV-4-infected mice showed splenomegaly and lymphadenopathy (Nash et al., 2001). The same was observed here. Myodes spleens are naturally much smaller than those of mice, but overall similar changes could be observed. Histological examination of mouse SCLNs at day 21 p.i. showed enlargement with increased numbers of germinal centres [Fig. 6b(ii, iv)]. Although bank vole SCLNs were luciferase-negative and yielded few observable infectious centres, viral antigens were more readily detectable than in mice [Fig. 6b(xii); 18.0±9.8 positive cells per field in Myodes vs 3.8±1.6 and 3.2±1.2 positive cells per field in BALB/c and CD1 mice, respectively; P<0.05 by one-way ANOVA and Bonferroni’s multiple comparison test].

**Antibody response analysis**

Lower viral loads in bank voles might be expected to induce weaker antibody responses. Sera taken at various times p.i. were analysed by a plaque reduction assay to determine the titres of neutralizing antibodies (Fig. 7). As viral neutralization assays can be influenced by the cell types on which they are performed, we repeated the experiment on three cell types from different origins: BHK-21 cells, derived from hamsters, and mouse NAMRU mammary gland (NMuMG) cells and 3T3 cells representing epithelial and fibroblastic murine cells, respectively. In each cell type, MuHV-4-specific neutralizing titres were low at day 7 p.i. in all animals, but increased dramatically by day 14. For some time points, the titres measured in bank voles were significantly lower than in mice (Fig. 7a–c).

**MuHV-4 is not transmitted between mice or voles in captivity**

Finally, we evaluated the ability of MuHV-4 to undergo transmission between female mice or voles maintained in captivity. In each group, three 6–8-week-old females were inoculated intranasally (10⁴ p.f.u. in 30 µl). At 2 days p.i., three naïve females were placed in the same cage as the inoculated animals. Luciferase signals were monitored once a week (data not shown) and sera were taken at various
times p.i. and analysed by a plaque reduction assay to
determine the titres of neutralizing antibodies (Fig. 8a).
Although all infected animals showed luciferase signals
characteristic of the MuHV-4 in vivo cycle and developed
neutralizing antibodies, none of the contact animals
presented any luciferase signal or neutralizing antibody. Finally,
almost all the animals were sacrificed at day 28 and latent virus
loads in spleens were quantified by real-time PCR (Fig. 8b).
Again, viral DNA was only recovered from infected
individuals and not from any of the contact individuals.

**DISCUSSION**

Co-speciation has evidently been the prominent mode of
evolution in the order *Herpesvirales* (Davison, 2002;
McGeoch, 2001; McGeoch *et al.*, 2000, 2006). In nature,
most herpesviruses are effectively closely associated with
a single host species. They have evolved over long periods of
time with their host and are extremely well-adapted to
them. This view is supported not only by phylogenetic
studies, but also by the modest pathogenicity of herpes-
viruses in their natural settings (Davison, 2002). In con-
trast, herpesvirus infections resulting from trans-species
transmission are generally associated with severe diseases
(Dewals *et al.*, 2006; Fickenscher & Fleckenstein, 2001; Hart
*et al.*, 2007). We have been studying MuHV-4 in mice. The
power of MuHV-4 to harness mouse genetic and immuno-
logical tools makes it an obvious choice for initial analysis.
Indeed, we recently described several immune-evasion
mechanisms that could explain why the antibody response
to natural infection does not prevent its transmission (Gillet

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**Fig. 4.** Classical analysis of MuHV-4 strain MHV-68 infection in *Myodes glareolus* (△) and BALB/c (□) and CD1 (◇) mice. Ten
animals (five males and five females) were infected intranasally (10⁴ p.f.u. in 30 µl) under general anaesthesia with wild-type
MuHV-4 strain MHV-68. Each horizontal line shows the mean for each group of ten (including negatives) and each point shows
the signal for one entire organ. (a) At 7, 14 and 21 days p.i., the infectious virus titre in noses was determined by plaque assay.
The titres at day 7 p.i. in *Myodes glareolus* noses were reduced significantly relative to those in BALB/c mice (P<0.001 by two-
way ANOVA and Bonferroni post-test), but not relative to those in CD1 mice (P>0.05). (b) At 7, 14 and 21 days p.i., the
infectious virus titre in lungs was determined by plaque assay. The titres in *Myodes glareolus* lungs were reduced significantly at
day 7 p.i. relative to those in BALB/c mice (P<0.001), but not relative to those in CD1 mice (P>0.05). (c) Superficial cervical
lymph nodes were removed at the indicated times and assayed individually for reactivatable MHV-68 by infectious centre assay.
The titres in *Myodes glareolus* SCLNs were only reduced significantly at day 14 relative to those in CD1 mice (P<0.001).
(d) Spleens from the same animals were analysed individually by infectious centre assay. The titres in *Myodes glareolus* spleens
were reduced significantly at day 14 relative to those in BALB/c and CD1 mice (P<0.05 and P<0.001, respectively), but not at
days 7 and 21.
Recently, two gammaherpesvirus infection models were described in wood mouse (A. sylvaticus) (Hughes et al., 2010a, b). In the first study, the authors characterized a novel wood mouse virus related to MuHV-4 (Hughes et al., 2010b). However, wood mouse herpesvirus and MuHV-4 share only 85% nucleotide sequence identity and therefore cannot be seen as strains of the same species. Although wood mouse herpesvirus seems to be a very interesting model, it will be very difficult to transpose all of the knowledge accumulated about MuHV-4 infection to this new model. In a second study, Hughes et al. (2010a) described MuHV-4 infection in the wood mouse. Based on PCR results, Blasdell et al. (2003) proposed MHV-68 as a wood mouse virus (Blasdell et al., 2003). However, they did not provide any sequence analysis. Moreover, subsequent data from the same group strongly suggested that they were amplifying a related virus rather than MHV-68 (Ehlers et al., 2008). This was reinforced by their recent paper describing wood mouse herpesvirus (Hughes et al., 2010b).

Therefore, although their characterization of wood mouse infection by MuHV-4 is interesting, there is major evidence suggesting that wood mice cannot be considered a natural host of MHV-68, only of the wood mouse virus. As MHV-68 has been isolated from bank voles (Blaskovic et al., 2010a, b), analysis of the MuHV-4 in vivo cycle in this species was therefore needed.

Intranasal virus inoculation led to productive replication in bank vole lungs, as is observed in mice; however the extent of lytic replication was approximately 1000-fold lower in bank voles than in mice. This observation could be related to the fact that the nose – but not the lung – is the most likely point of normal host entry for MuHV-4 (Milho et al., 2009). Indeed, intranasal inoculation of mice without anaesthesia gave luciferase expression in just the nose and not in the lung, with a normal latent colonization of draining lymph nodes. In this study, despite lower replication levels in the lung, we observed similar latent loads in voles and mice (Fig. 5). MuHV-4 host colonization is relatively independent of the extent of primary lytic infection (Coleman et al., 2003; Stevenson et al., 1999). It depends much more on latency-associated lymphoproliferation (May et al., 2004). As gammaherpesvirus epidemiology indicates that transmission correlates with the latent load, our results suggest that gammaherpesviruses may have evolved to infect their hosts without extensive lytic spread, which could provide a powerful immune stimulus. Besides a lower extent of lytic replication, Hughes et al. (2010a) reported focal granulomatous infiltrations in Apodemus lungs, rather than diffuse lymphocytic interstitial pneumonitis as observed in mice. Our experiments in Myodes, however, revealed lung lesions comparable to those observed in mice (Fig. 6a). Although lytic replication was limited in Myodes lungs, infection was accompanied by a characteristic diffuse interstitial pneumonitis. In the future, it would be interesting to see whether similar pathological changes are observed in mice after intranasal inoculation without anaesthesia. In comparison with

Fig. 5. Latent loads in spleens and SCLNs of Myodes glareolus (filled bars) and BALB/c (empty bars) and CD1 (grey-shaded bars) mice. The same samples as in Fig. 4(c, d) were analysed for viral genomes by real-time PCR of DNA from spleens or SCLNs. Each bar shows the mean viral genome copy number per host genome ± SEM for each group of ten. The dashed lines show the lower limits of assay sensitivity. (a) At day 7, viral genome loads in Myodes glareolus spleens were not significantly different from those measured in mice (two-way ANOVA and Bonferroni post-test). However, they were reduced significantly at days 14 and 21 relative to BALB/c but not CD1 mice (P<0.01 and P<0.001, respectively). (b) Viral genome loads in Myodes glareolus SCLNs were not significantly different from those measured in mice (two-way ANOVA and Bonferroni post-test).

All viruses must be selected primarily for efficient transmission. Failure of experimental transmission in the mouse could be due to several reasons, among which is the fact that the house mouse (Mus musculus) may not be a natural host for MuHV-4. Although there are serological indications that MuHV-4 (or a close relative) naturally infects house mouse (Mistrikova et al., 2000), the virus has never been isolated from this species. We therefore do not have any idea whether it is a relevant model. Comparisons of MuHV-4 infection in mice and natural hosts could provide answers to that question.
laboratory mice, the spleens of infected bank voles showed reduced splenomegaly. However, long-term latency was maintained similarly in voles and mice, suggesting that splenomegaly is not required for long-term latency. Finally, an intriguing observation was that, although bank vole SCLNs were luciferase-negative and yielded few observable

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**Fig. 6.** Tissue histology and immunohistochemistry. (a) At 7 days after infection with wild-type MuHV-4 strain MHV-68, the lungs of *Myodes glareolus* and BALB/c and CD1 mice were removed and fixed in formaldehyde before haematoxylin/eosin staining. In the three species, lung infection evoked interstitial pneumonia with massive cellular infiltrates and oedema (i–vi). Samples from the same organs were then processed for immunohistochemistry and stained with anti-MHV-68 rabbit polyserum (vii–xii). The results confirmed that viral pulmonary replication was much lower in *Myodes glareolus* than in BALB/c and CD1 mice. The arrow indicates focal MuHV-4 antigen detection. The images are representative of data from at least five animals. (b) At 21 days after infection with wild-type MuHV-4 strain MHV-68, SCLNs of *Myodes glareolus* and BALB/c and CD1 mice were removed and fixed in formaldehyde before haematoxylin/eosin staining (i–vi). An increased number of germinal centres was observed mainly in BALB/c and CD1 mice. Samples from the same organs were then processed for immunohistochemistry and stained with anti-MHV-68 rabbit polyserum (vii–xii). The arrows indicate representative focal MuHV-4 antigen detection. The images are representative of data from at least five animals.
infectious centres, viral antigens were more readily detectable than in mice. Several hypotheses can explain this observation, such as differences in tropism, differences in spreading and differences of antigenicity of some viral proteins. The significance of this phenomenon therefore remains unclear and will require further experiments in the future to be understood.

The main features of MuHV-4 infection in Myodes glareolus are very similar to those observed in A. sylvaticus. However, unlike what we found in Myodes, Hughes et al. (2010a) described titres of neutralizing antibody to MuHV-4 that were significantly higher in wood mice than in BALB/c mice. They related this observation to the histological changes that they found and that we did not observe in Myodes glareolus. Whilst this is possible, another explanation could be that their results mainly reflected poor neutralizing antibody titres in BALB/c mice, although numerous studies have investigated MuHV-4 neutralization in this model. This difference could be due to the cell type they used to perform their experiments, as every laboratory 3T3 cell line is potentially a bit different. For example, the mouse cells that they used could have provided Fc receptors that could have reduced BALB/c serum neutralizing activity (Rosa et al., 2007). Other effects of wood mouse sera on 3T3 cells are also possible. We therefore performed our plaque reduction assays on three cell types from mouse and hamster origins. In each cell type, we observed neutralizing antibody titres in Myodes that were similar overall to those observed in mice, even if they tended to be lower at some time points. Further comparative studies on both Myodes and Apodemus sera in parallel are therefore needed to assess the differences between MuHV-4 neutralizing antibody responses in Myodes glareolus and A. sylvaticus.

The tendency towards lower antibody titres in Myodes glareolus suggests that transmission could be easier in bank voles than in mice. We therefore created epidemiological situations by mixing MuHV-4-infected and naïve animals. We monitored possible transmission for 1 month by serology, in vivo bioluminescence and genome quantification in spleens. However, it was impossible to establish experimental transmission in populations of either mice or voles. Several hypotheses could explain this lack of transmission. Firstly, these results could reflect the fact that gammaherpesvirus transmission is a rare event. However, the prevalence of these viruses in natural populations suggests the opposite. Secondly, luciferase expression could prevent virus transmission by altering the long-term behaviour of the virus, as recently shown by a MuHV-4 strain expressing the non-structural protein NS3 of hepatitis C virus under the control of the murine cytomegalovirus promoter (MHV-68-NS3; El-Gogo et al., 2008). To avoid this problem, luciferase was placed under the control of the MuHV-4 M3 promoter and therefore was only expressed in lytically infected cells. In contrast to MHV-68-NS3, no decreased viral latent load was observed for this strain in comparison with the wild-type strain (Milho et al., 2009). However, further studies with the wild-type MuHV-4 strain will be needed to determine whether luciferase expression during lytic replication could prevent virus

Fig. 7. Kinetics of the virus-specific neutralizing humoral response following infection with MuHV-4. Five females per group were infected intranasally (10⁴ p.f.u. in 30 μl) under general anaesthesia with wild-type MuHV-4 strain MHV-68 and blood samples were taken at the indicated times p.i. The neutralizing antibody titre was determined by plaque inhibition on BHK-21 (a), 3T3 (b) or NMuMG (c) cells (see Methods). All measurements were made relative to a standard pool of immune sera and the reciprocal of the dilution that resulted in a >50% reduction in the number of MuHV-4 plaques was plotted. Empty bars, BALB/c; grey-shaded bars, CD1; filled bars, Myodes glareolus. Each bar shows the mean ± SEM for each group of five. Asterisks indicate the values for each day that are significantly different from the Myodes values: *P<0.05, ***P<0.001 by two-way ANOVA and Bonferroni post-test.
transmission in *Myodes glareolus* populations. Thirdly, failure of experimental transmission could be because the transmission conditions that we used were not appropriate. MuHV-4 and its close relatives are, unusually, found in a wide variety of hosts (mouse, shrew and bank vole). We are used to thinking of herpesviruses as being highly host-restricted (McGeoch et al., 2006). However, recent phylogenetic analysis has shown that for some gammaherpesvirus species, including MuHV-4, distant interspecies transfer has been an important part of their evolutionary history (Ehlers et al., 2008). We can assume that there may be more horizontal transmission between small rodents than between large mammals, simply because more species share overlapping ecological niches. This diversity of hosts could explain why MuHV-4 genomes have been accumulating sequence changes atypically fast compared with other members of the *Gammaherpesvirinae* (McGeoch, 2001; McGeoch et al., 2005). Furthermore, nose infection has raised the possibility of transmission through scent marking. This behaviour is distorted in caged mice. Therefore, we need to test MuHV-4 transmission between *Mus musculus* in a more physiological context than conventional animal caging. Another way would be to test MuHV-4 transmission in another described host species. However, it is unclear whether the infections observed in field studies are always productive. MuHV-4 (or a related virus) has been isolated repeatedly from yellow-necked mice (*A. flavicollis*), so these could be the natural host. In the future, testing whether they transmit in captivity would be an interesting point.

In conclusion, MuHV-4 infection of bank voles follows the same route as in mice, but the virus replicates to a lesser degree. These differences in the extent of lytic replication could just mean that strain MHV-68 is better adapted to replication in mice. An MHV-68 isolate has actually been obtained from successive intracranial passages in newborn mice (Blaskovic et al., 1980; Nash et al., 2001). By contrast, limited lytic replication with normal long-term latent loads could also represent better adaptation of the virus to its host, as is also observed for wood mouse herpesvirus in the wood mouse (Hughes et al., 2010b). In the future, we hope to be able to choose between these hypotheses in light of virus transmission. Whilst MuHV-4 infection in yellow-necked mice – another species known to be naturally infected – may yet yield surprises, it appears so far that *Mus musculus* represents a suitable host for studying gammaherpesvirus pathogenesis with MuHV-4.

**Fig. 8.** Transmissibility of MuHV-4 in *Myodes glareolus* and BALB/c and CD1 mice. Three females per group were infected intranasally (10⁴ p.f.u. in 30 μl) under general anaesthesia with wild-type MuHV-4 strain MHV-68. At 48 h p.i., three naïve females (contact) were placed in the same cage as the inoculated animals. (a) Blood samples were taken at the indicated times p.i. The neutralizing antibody titres were determined by plaque inhibition on BHK-21, 3T3 or NMuMG cells (see Methods). All measurements were made relative to a standard pool of immune sera and the reciprocal of the dilution that resulted in a >50% reduction in the number of MuHV-4 plaques was plotted. Empty bars, contact animals; filled bars, infected animals. Each bar shows the mean ± SEM for each group of three. (b) The animals were sacrificed at day 28 p.i. and their spleens were analysed for viral genomes by real-time PCR. Each bar shows the mean viral genome copy number per host genome ± SEM for each group of three.
METHODS

**Animals.** Female and male BALB/c and CD1 mice were purchased from Charles River Laboratories. We used adult female and male bank voles from generation 2 of a colony established from voles maintained in a large experimental colony in Poland (Sadowska et al., 2008). Their offspring (generation 1) were weaned at day 21 and maintained individually in standard polypolyethylene mouse cages (26 × 20 × 16 cm). Voles from generation 1 were paired at the age of 4–6 months to produce generation 2 (some individuals in generation 2 were cousins or paternal half-siblings). All the animals were housed in the Department of Infectious Diseases, University of Liège. The animals were infected with MuHV-4 at 6–12 weeks old. Intranasal infections with anaesthesia were in 30 μl aliquots. For luciferase imaging, animals were anaesthetized with isoflurane, injected intraperitoneally with d-luciferin (150 mg kg⁻¹), then scanned with an IVIS Spectrum (Caliper Life Sciences). Animals were routinely imaged after 10 min. For quantitative comparisons, we used Living Image software (Caliper Life Sciences) to obtain either the maximum radiance [photons (p) s⁻¹ cm⁻² per steradian (sr)] or total flux (p s⁻¹) over each region of interest. All experiments conformed to the rules of the local animal ethics committee of the University of Liège.

**Cells and virus.** BHK-21, NMuMG and 3T3 cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen), supplemented with 2 mM glutamine, 100 U penicillin ml⁻¹, 100 mg streptomycin ml⁻¹ and 10 % FCS. We used the wild-type MHV-68 strain of MuHV-4 (Blaskovic et al., 1980). The MuHV-4 strain expressing luciferase under the control of the M3 promoter has been described previously (Milho et al., 2009).

**Viral infectivity assays.** Virus stocks were titrated by plaque assay on BHK-21 cells (Gillet et al., 2007b). Cell monolayers were incubated with virus (2 h, 37 °C), overlaid with 0.3 % carboxymethylcellulose, and 4 days later were fixed and stained for plaque counting. Infectious virus in lungs was measured by homogenizing the lungs in 6 ml complete medium prior to plaque assay. To determine nasal titres, we removed a block of tissue bound (i.e. not including) anteriorly by the cartilaginous tip of the nose, posteriorly by the orbits, laterally by the zygomatic arches, ventrally by the palate and dorsally by the nasal bones. This region contained all the luciferase signal measurable by ex vivo CCD camera scanning. Bone fragments were discarded after homogenization in 3 ml complete medium and the lysate was plaque assayed. Latent virus in tissues was quantified by hybridization with a TaqMan probe (genomic co-ordinates 4218–4189, 5′-FAM-TCCAGCCATTCCTAGGCTCCTCTAATGA-BHQ1-3′) and converted to genome copies by comparison with a standard curve of cloned plasmid template serially diluted in control spleen DNA and amplified in parallel. Cellular DNA was quantified in parallel by amplifying part of the interstitial retinoid binding protein (IRBP) gene (forward primer 5′-ATCCCATGATCCTCCTTG-3′, reverse primer 5′-CCCTGTGCTTCCAGTGTT-3′). The PCR products were quantified with SYBR Green (Invitrogen) and the copy number was calculated by comparison with standard curves of cloned IRBP templates from each species amplified in parallel. Amplified products were distinguished from paired primers by melting curve analysis, and the correct sizes of the amplified products were confirmed by electrophoresis and staining with ethidium bromide.

**Lung histology and immunohistochemistry.** Portions of lungs and SCLNs were fixed in buffered formol saline, processed routinely to 5 μm paraffin wax-embedded sections, stained with haematoxylin and eosin, and examined by light microscopy. Immunohistochemistry was performed using an EnVision Detection System (Dako) with anti-MHV-68 rabbit hyperimmune serum (Sunil-Chandra et al., 1992) as the primary antibody.

**Measuring neutralizing antibody.** Duplicate twofold serum dilutions, starting from an initial concentration of 1:10 in DMEM containing 10 % FCS, were incubated with 40 p.f.u. MHV-68 at 37 °C for 1 h, in 96-well plates. Freshly trypsinized cells (2 × 10⁴) were added to each well and allowed to adhere for 2 h. The cells were then overlaid with 0.3 % carboxymethylcellulose, and 4 days later fixed and stained for plaque counting. A standard immune serum was included in each experiment to ensure uniformity of results. The neutralization titre was defined as the highest serum dilution giving a >50 % reduction in viral plaques. Naïve mouse and *Myodes* sera had no effect on plaque formation.

ACKNOWLEDGEMENTS

S.F. is a Research Fellow of the Belgian ‘Fonds pour la formation à la Recherche dans l’Industrie et dans l’Agriculture’. S.V. and L.G. are Research Fellow and Research Associate of the ‘Fonds National Belge de la Recherche Scientifique’ (FRS-FNRS), respectively. P.G.S. is a Welcome Trust Senior Clinical Fellow (GR076956MA). This work was supported by the following grants: starting grant of the University of Liège (D-09/11) and scientific impulse grant of the FRS-FNRS no. F.4510.10.

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