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A novel small animal model to study the replication of simian foamy virus *in vivo*

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ABSTRACT

Preclinical evaluation in a small animal model would help the development of gene therapies and vaccines based on foamy virus vectors. The establishment of persistent, non-pathogenic infection with the prototype foamy virus in mice and rabbits has been described previously. To extend this spectrum of available animal models, hamsters were inoculated with infectious cell supernatant or bioballistically with a foamy virus plasmid. In addition, a novel foamy virus from a rhesus macaque was isolated and characterised genetically. Hamsters and mice were infected with this new SFVmac isolate to evaluate whether hamsters are also susceptible to infection. Both hamsters and mice developed humoral responses to either virus subtype. Virus integration and replication in different animal tissues were analysed by PCR and co-cultivation. The results strongly indicate establishment of a persistent infection in hamsters. These studies provide a further small animal model for studying FV-based vectors in addition to the established models.

Introduction

Foamy viruses are complex retroviruses that are presently the only known members of the genus *Spumavirus*. Spumaviruses have been isolated from several mammals, particularly horses, cats, cattle, non-human primates and apes, with a high prevalence in adult captive primates. Despite long periods of persistent infection, no signs of disease or pathological changes have ever been observed (Blewett et al., 2000; Falcone et al., 2003; Schweizer et al., 1995). Today, it is generally agreed that humans are not a natural host for foamy viruses (Heneine et al., 1998; Herchenroder et al., 1994). Furthermore, disease has never been observed in any of the known cases of zoonotic transfer of SFV to humans (Boneva et al., 2007). This makes foamy viruses interesting candidates for the development of vector systems for gene therapy (Erlwein and McClure, 2010). In addition, the life-long chronic infection of the host offers an advantage for the development of novel vaccines able to induce a persistent, targeted and effective stimulation of the immune system using replication-competent vaccine viruses.

There are basically two small animal models currently available for studying foamy virus infection. The first involves intraperitoneal (i.p.), intradermal or intranasal (i.n.) inoculation of rabbits with SFV-infected cell culture supernatant, which leads to a persisting infection that can be detected for at least 264 days by co-cultivating cells from various organs (Swack and Hsiung, 1975). In one experiment, an immune response to viral antigens could be demonstrated five years after infection (Saib et al., 1997). The second model involves SFVcpz infection of mice, demonstrated by virus re-isolation and detection of specific antibodies. Brown et al. (1982) showed virus replication in the spleen and kidneys, although as time progressed this replication became increasingly difficult to demonstrate. In later experiments it was possible to demonstrate virus replication in various mouse strains by detection of provirus by PCR and the measurement of an SFVcpz(hu) specific immune response, although virus re-isolation was successful in only a few cases (Schmidt et al., 1997b). Again, infection was not associated with any obvious signs of disease. However, some studies have suggested an immunosuppressive effect during the initial phases of an SFVcpz(hu) infection of rabbits and mice that was subsequently compensated for by the immune system as the study progressed (Hooks and Detrick-Hooks, 1979; Santillana-Hayat et al., 1993) although no other effect on the animal's health was observed and the immunological changes cleared over time. Transgene mice that permanently express FV antigens in all cells do show damage to the central nervous system

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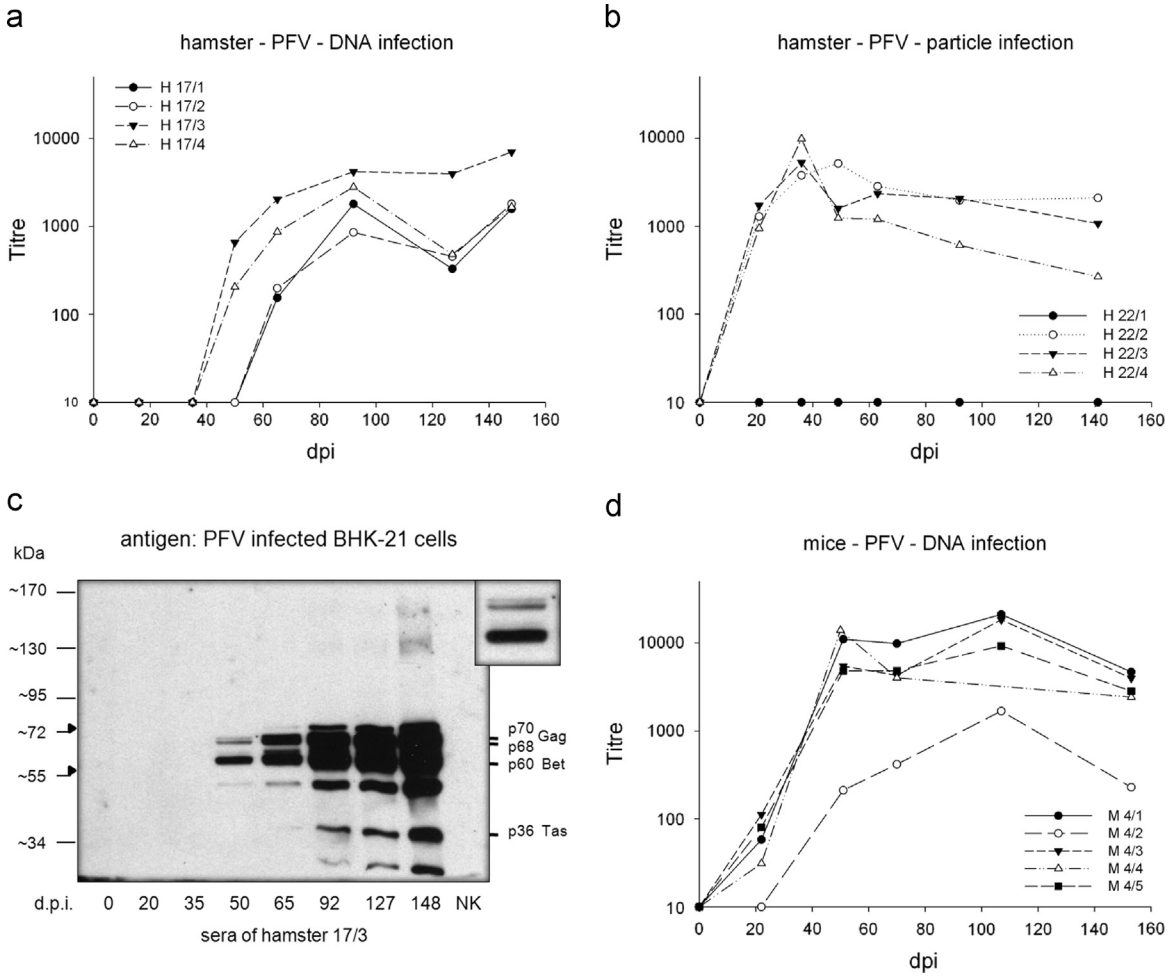

Fig. 1. (a) Development of PFV-specific antibodies in hamsters following gene gun inoculation of 3 μg pHSRV2/13 DNA. (b) Development of PFV-specific antibodies in hamsters following inoculation of 10^6 infectious PFV particles. PFV-infected BHK-21 cell lysate was used to coat the ELISA plates. Sera were diluted in three-fold steps from 1:20 to 1:43,740 and the titre calculated as the dilution at which the best-fit curve intercepted the cutoff OD (0.2). (c) Western blot of PFV-specific antibody development in hamster H17/3 (infected by gene gun DNA delivery). PFV-infected BHK-21 cells lysed with RIPA-buffer were used as antigen and sera were diluted 1:750. In order to recognise the processing of the Gag precursor (p70.6 kDa) into p68 Gag, the relevant region of the blot (indicated in the size ladder by arrows) is shown enlarged. The right box shows the PFV-Gag double bands and lower Bet band from lane 4. No cross-reaction could be detected against BHK naive lysate (NK). The putative PFV-protein bands p70/68 Gag, p60 Bet and p36 Tas (Lindemann and Goepfert, 2003) are labelled. (d) Development of PFV-specific antibodies in mice following gene gun inoculation of PFV-DNA. ELISAs were performed as described above.

and striated muscle (Aguzzi et al., 1996). Although this highly artificial situation suggests a pathogenic potential for foamy virus antigens when expressed at high levels, such effects have never been seen in infected natural hosts or in the documented cases of zoonosis.

This paper describes a new small animal model for studying foamy virus infection *in vivo* that uses Syrian golden hamsters. The hamster was long ago reported to be a natural host for foamy viruses (Hruska and Takemoto, 1975) although there are no later publications confirming the existence of a hamster foamy virus, which calls the initial report into question. Certainly, no evidence (by serology, PCR or co-cultivation) for a pre-existing natural infection could be found in the Syrian golden hamsters (Charles River, Sulzfeld, Germany) involved in the study presented here.

We could, however, show that Syrian golden hamsters are susceptible to infection with a range of different foamy virus isolates. Furthermore, we describe a novel method of foamy virus infection involving inoculation of the animals via gene gun with a purified molecular clone of the PFV genome. The development of the antibody response to viral antigens during the early phase of infection is described and the new hamster model is compared to the currently established mouse model.

Results

Inoculation of animals with PFV

To facilitate the study of the foamy virus infection *in vivo* we attempted to establish a new small animal model using Syrian golden hamsters. Hamsters and C57BL/6 mice were inoculated in parallel to allow the new model to be compared with the published mouse model (Schmidt et al., 1997b). The molecular clone pHSRV2/13 (kindly provided from Martin Löchelt, DKFZ Heidelberg) used here for the infection studies contains a deletion in the U3 region but has replication characteristics similar to those of a comparable wild-type virus. Its use also allows a direct comparison to be made with the results from previous publications that have predominantly used this variant (Nestler et al., 1997; Saib et al., 1997; Schmidt and Rethwilm, 1995), including that of Schmidt et al. (1997b) describing the mouse model.

In addition, inoculation with naked plasmid DNA via gene gun (GG) was compared with the usual method that uses infectious cell culture supernatant. Initiation of infection using DNA is a relatively novel technique that offers a number of advantages (*e.g.* genetic stability, no contamination with cell culture material, good

reproducibility) over traditional methods and has been used to initiate infection (Kent et al., 2001). DNA corresponding to the foamy virus molecular clone pHSRV2/13 (Löchelt et al., 1991; Schmidt et al., 1997a) was inoculated by gene gun into the upper epidermis. To allow a direct comparison with the standard method of inoculating infectious virus particles, a group of four hamsters was inoculated i.p. with 1 ml cell culture supernatant containing approximately 10^6 infectious PFV particles. The animals were inspected every day for external signs of pathology and other signs of disease but no such symptoms were observed during the course of the study.

Development of antibody responses to PFV in hamsters and mice

Retrobulbar blood samples were taken from hamsters and mice over a period of 148 days post inoculation [d.p.i.]. Serum IgG specific for PFV proteins was detected by ELISA and western blot. Lysates of PFV-infected and uninfected BHK-21 cells were used as antigen. Fig. 1 a, b and d shows the development of PFV-specific antibodies after inoculation as measured by ELISA. Hamsters inoculated with purified PFV-DNA showed no signs of a humoral immune response in the first 35 days following application of the DNA but a continuous increase in responses from this time point until day 92 (Fig. 1a). This was followed by a temporary drop in antibody levels as measured on day 127 before a recovery or even increase in levels thereafter. This pattern of reactivity suggests a persisting infection with possible repeating phases of viraemia. The ELISA data could be confirmed by western blot. Fig. 1c shows, for example, the antibody response to the typical PFV antigens in hamster 17/3 over time. No reactivity can be seen on days 20 and 35 after inoculation, with the first positive signals (to the viral Gag and Bet proteins) being seen on day 50 and increasing in the subsequent samples. The p70/p68 double band typical for foamy viruses (area indicated by arrows) is shown enlarged in Fig. 1c (right box, top) as the small difference in molecular weight causes the two bands to run very close to each other. The bands below the double bands probably represent the p60 Bet protein. The viral proteins Gag, Bet and Tas have been labelled according to the calculated molecular weights. The remaining bands presumably represent the proteins gp170Env-Bet, gp130Env, gp80SU and gp48TM (Lindemann and Goepfert, 2003). Bet and Gag appear to be the immunodominant proteins, inducing in the early stages the strongest immune responses. The specificity of the immune responses observed is demonstrated by the lack of binding using pre-immune serum (Day 0) and the lack of response to uninfected BHK-21 cell lysate (NK). The other animals in the group showed similar patterns of western blot activity (data not shown).

To address the possibility that the immune responses observed in hamsters were simply the result of 'genetic immunisation' (*i.e.* through expression of viral proteins from inoculated DNA without infection) we constructed a replication-deficient HSRV2/13 viral genome by deletion of the complete bel2-ORF including the 3′ polypurine tract in front of the 3′LTR. After confirming lack of infectivity in cell culture, a group of four hamsters was inoculated via gene gun. Blood samples were taken over a period of 105 days and sera subjected to ELISA. As shown in Fig. 2 of the supplement, antibodies specific for PFV were not detected.

The development of PFV-specific antibodies in hamsters inoculated with virus-containing cell culture supernatant is shown in Fig. 1b. The pattern of reactivity is clearly different than that seen with inoculation via DNA, indicating possible differences in the initial infection. Sera from three of the four animals contained PFV-specific antibodies at 20 days post inoculation that peaked at 35–50 days and then remained constant or diminished thereafter. Animal H22/1 remained seronegative by ELISA and western blot (WB data not shown).

The development of PFV-specific antibodies in mice inoculated via gene gun was also measured by ELISA (Fig. 1d). In contrast to the DNA-inoculated hamsters, the mice generally showed signs of a humoral immune response at 22 days post inoculation. These levels peaked between 51 and 107 days and by day 148 antibodies in all animals were decreased. However, the antibody levels were generally much higher compared to the hamster sera. One mouse (4/2) showed a weaker reaction to PFV inoculation. These data suggest that, in C57BL/6J mice, DNA-inoculation with PFV stimulates the immune system differently than in hamsters. The stronger immune response may result in a more vigorous suppression of viraemia leading to a decrease in antibody levels as antigenic stimulation wanes.

Isolation and characterisation of a new SFVmac isolate from rhesus macaques

We tested next the replication competence of additional primate foamy virus strains in these animals. A new strain of SFVmac was isolated from a rhesus macaque housed at the Paul Ehrlich Institute (Langen, Germany). Fresh PBMC were co-cultivated with BHK-21 cells and after three weeks the typical cytopathic effect (CPE) was observed in the cell culture. In order to precisely characterise the newly isolated virus strain, the complete genome was sequenced and compared with those of other known foamy viruses. The ORFs for Gag, Pol and Env as well as the ORF1 and ORF2 for the accessory proteins Tas and Bet agree with those described by Kupiec et al. (1991) for the published SFV1 isolate (NCBI: X54482.1). The isolated virus could be shown to clearly belong to the SFVmac class, albeit with a divergence of 12% from the published sequence. The individual open reading frames of the viral genome were also compared to the sequences of the SFV1 and PFV (HSRV2) isolates described above and the theoretical sizes of the precursor proteins calculated (Table 1). It is presently not possible without further investigation to draw any conclusions concerning the processing of the individual proteins. However, the sequences do contain protease cleavage sites typical for foamy viruses, suggesting a corresponding form of processing. The new isolate was named SFVmac-R289hybAGM and the sequence submitted under reference number JN801175.1 to the NCBI. Interestingly, a closer analysis of the Env ORF sequence reveals an anomaly that was also present in an SFVmac isolate stored at ATCC (Galvin et al., 2013). A higher divergence was found in the SU region of the new isolate (less than 60% homology between amino acids 242 and 505 of Env). A new analysis concentrating on this region revealed a 76% homology at the amino acid level with a simian foamy virus strain of the AGM class (Schweizer et al., 1999). For this reason, the isolate was termed 'hybrid AGM'. It is possible that this virus was newly formed by recombination in a host cell superinfected with two distinct viruses. Such a recombination in the envelope gene has been recently described (Galvin et al., 2013). A detailed analysis of the precise characteristics of the newly described virus and the impact of the presumed recombination event will be carried out in the future. Initial *in vitro* studies do, however, indicate an advantage with regard to replication efficiency (data not shown).

Development of SFVmac-R289hybAGM specific antibodies in hamsters and mice

The isolated SFVmac was then characterised *in vivo*. As the new virus was not available as a recombinant DNA genome at the time of starting the experiment, it was not possible to infect animals by gene gun. The groups of mice and hamsters were therefore each inoculated intraperitoneally with 1 ml of cell culture supernatant containing approximately 10^6 infectious SFVmac-R289hybAGM particles. Throughout the study, blood samples were taken regularly and the

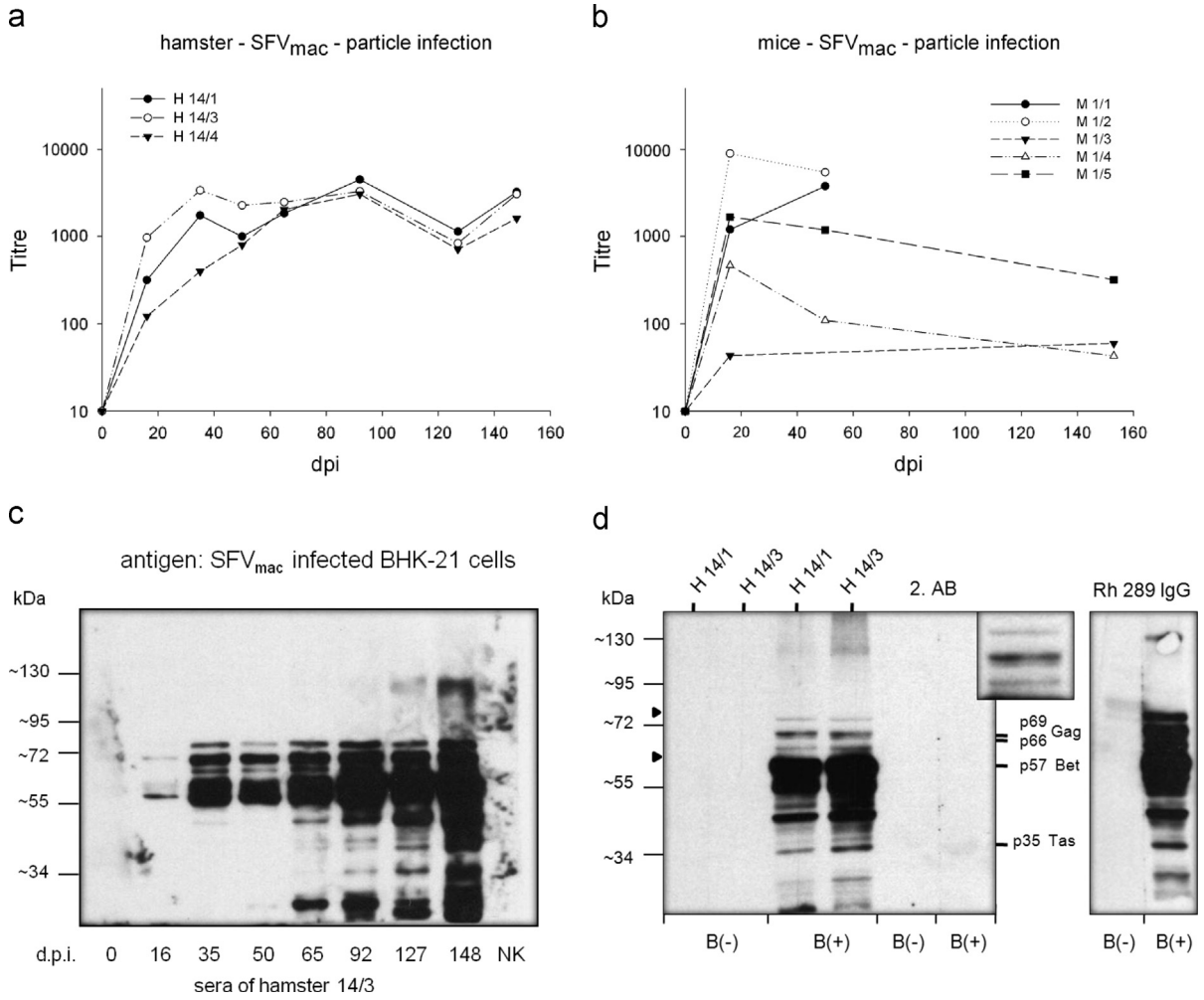

Fig. 2. (a) Development of SFVmac-R289hybAGM specific antibodies in hamsters following inoculation with 10^6 infectious particles. R289hybAGM-infected BHK-21 cell lysate was used to coat the ELISA plates. Sera were diluted in three-fold steps from 1:20 to 1:43,740 and the titre calculated as the dilution at which the best-fit curve intercepted the cutoff OD (0.2). (b) Development of SFVmac-R289hybAGM-specific antibodies in mice following inoculation with 10^6 infectious particles. ELISAs were performed as described above. (c) Western blot of SFVmac-specific antibody development in hamster H14/3. SFVmac-R289hypAGM-infected BHK-21 cells lysed with RIPA-buffer were used as antigen and sera were diluted 1:750. No cross-reaction could be detected against BHK naive lysate [NK]. (d) Western blot control for the specificity of IgG developing in the SFVmac infected hamster compared to the response of the macaque from which the virus was originally isolated. Sera from the SFVmac infected hamster were tested on uninfected BHK-21 [B(−)] and SFVmac infected BHK-21 lysates [B(+)] at a serum dilution of 1:1000. No cross-reaction of the secondary antibody with the cell lysate was observed (lanes 5 and 6). The small box shows an enlargement of the SFVmac Gag double-band from lane 3 (indicated by arrows in the MW ladder). The putative bands for p69/p66 Gag, p57 Bet and p35 Tas (Lindemann and Goepfert, 2003) are labelled. The IgG response of the naturally infected rhesus macaque 289 to SFVmac proteins is shown on the right. No cross-reactivity to uninfected BHK-21 lysate could be detected. Sera were diluted 1:1500 .

Table 1
Predicted molecular weights of SFVmac-R289hybAGM precursor proteins (without glycosylation) and sequence homology to other foamy viruses.

SFVmac-R289hybAGM	LTR	Gag	Pol	Env	Tas	Bet
kDa	–	68.9	130.1	112.8	35.1	57.4
SFV1[a]	89%	88%	96%	82%	82%	79%
PFV[b]	81%	47%	79%	68%	39%	37%

[a] Indicates homology at the DNA level between SFVmac-R289hybAGM and SFV1 sequence (NCBI: X54482).
[b] Indicates homology at the DNA level between SFVmac-R289hybAGM and the PFV sequence (HSRV2; NCBI: Y07724).

sera tested for SFVmac-specific antibodies using the established ELISA. As one hamster died during the initial blood sampling, it was only possible to obtain data for three animals of the SFVmac group. The results from the infected hamsters are shown in Fig. 2a. SFVmac specific antibodies were first detected shortly after the inoculation, indicating a strong initial infection. The responses increased steadily thereafter reaching an initial peak on day 92 before showing a sudden drop and subsequent recovery. This pattern of reactivity again suggests a persistent infection with viraemic phases. The ELISA data for the three animals could be confirmed by western blot. Fig. 2c shows, for example, the clear increase in the levels of antibodies from animal 14/3 able to bind to viral antigens in western blot. Specific antibodies were first detected after 16 days. Specificity of the immune responses observed is demonstrated by the lack of binding of pre-immune serum (Day 0) and the lack of response to uninfected BHK-21 cell lysate. An additional western blot was performed to confirm the specificity of the antibodies. Sera from the final bleed of hamsters 14/1 and 14/3 were tested against uninfected and SFVmac infected BHK-21 cell lysates (Fig. 2d). No reaction with uninfected BHK cells was observed. A further specificity control with secondary antibody alone was also negative with both cell lysates. However, the separation of the p69/p66 Gag double band (area indicated by arrows) was very difficult to distinguish and this part of the blot is therefore shown enlarged. The bands p57 Bet and p35 Tas were also labelled. Classification of the addition bands is, due to the novelty of the isolate, presently not possible and will be the subject of future studies. To compare the

Table 2
PFV and SFVmac-R289hybAGM re-isolation from saliva, lung- and spleen-cells.

	Hamsters		Mice	
	PFV[a]	SFVmac[b]	PFV[a]	SFVmac[b]
Saliva	0/4	0/3	0/5	0/3
Lung	0/4	3/3	0/5	0/3
Spleen	0/4	1/3	0/5	0/3

[a] Animals were inoculated with 3 μg HRSV2/13 (PFV) DNA via gene gun (mouse group M4 hamster group H17).
[b] Animals were inoculated with 1ml SFVmac-R289hybAGM (10^6 infectious particles) containing supernatant (mouse group M1, hamster group H14).

immune response with that of the natural host, the antibody response of the animal from which the virus was originally isolated was tested using infected and uninfected cell lysates (Fig. 2d, right). Despite the monkey having a stronger reaction, the similarity in the pattern of bands suggests a comparable immune response. The lack of reactivity with uninfected BHK-21 cell lysate again confirms specificity. Fig. 2b shows the development of specific antibodies in the infected mice. Unfortunately, two of the animals died during the blood sampling on day 51, leaving only three to complete the study. To avoid further loss of animals, sampling was restricted to a final bleed on day 148. Similar to the situation with the PFV-DNA inoculated mice, a specific response was seen during the initial phase of infection. The drop in antibody response at the end of the experiment is also similar to that seen in PFV inoculated mice, again indicating different courses of infection in mice and hamsters. This late loss of antibodies was confirmed by western blot in which only weak bands at the level of p69/p66 Gag and p57 Bet were visible (data not shown).

Neutralising antibody responses in rhesus macaques and hamsters

An assay to evaluate the ability of FV-specific antibodies to neutralise virus infectivity was developed and used to test sera from naturally infected macaques and inoculated hamsters (Fig. 3 of the supplement). In comparison to the long-term infected rhesus monkeys, only a relatively weak neutralising effect could be demonstrated in the hamsters. Sera from all naturally infected rhesus macaques, when diluted 1:20, showed almost 100% neutralisation of SFVmac. Indeed, two macaques showed strong cross-neutralisation of PFV, whereas the other two animals had type-specific neutralising activity. As sera from the hamster group inoculated with replication-incompetent HSRV2/13 (group H3) showed no neutralising activity, the effect would appear to be specific. No cross-reactivity between the groups of inoculated hamsters was observed.

At the time of blood sampling, the rhesus macaques were at least two years SFV-positive, and probably much longer. This could account for the significantly higher titres and cross-reactivity. In addition, the difficulties in isolating virus from experimentally infected animals indicates that virus loads in hamsters and mice are much lower than in the natural host and this could result in weaker immune responses. The induction of neutralising antibodies nevertheless provides further evidence for a productive infection.

Re-isolation of PFV and SFVmac from hamsters and mice

Attempts to demonstrate virus replication in selected organs were made at the end of the infection studies at 148 d.p.i. Lungs and spleens of animals inoculated with SFVmac supernatant or via gene gun with PFV-DNA were removed and processed to yield single cell suspensions that were co-cultivated with MRC-5 cells. Because virus re-isolation using species-homologous cells can be inhibited by the release of gamma-interferon from infected blood cells (Falcone et al., 1999b), we always used species-mismatched cell lines for the co-cultures. Furthermore, the oral and nasal cavities of hamsters and mice were washed out with PBS that was then added to MRC-5 to check for viral replication in the oral mucosa. Co-cultures with lung cells from hamsters 14/1 and 14/4 showed typical CPE after two weeks incubation. Four days later, the spleen co-cultures from hamster 14/1 were also positive. The lung co-cultures from hamster 14/3 became positive after three weeks. The co-culture experiments were stopped after four weeks, the cells were lysed and the presence of proviral DNA tested by SFVmac- and PFV-specific PCR (Table 2). All cultures that showed CPE also tested positive for SFVmac provirus. Mouse organs tested negative for SFVmac and all PFV inoculated animals tested negative for provirus. Similarly, all co-cultures with mucosal washes remained negative for CPE and provirus.

Detection of proviral DNA in the organs of PFV and SFVmac infected animals by nested PCR

In addition to the co-culture experiments, the individual organs were tested for the presence of proviral DNA as evidence for productive infection. A nested PCR was developed that detected proviral DNA in the SFVmac/PFV integrase region and this was used to test genomic DNA isolated from the gut, lungs, liver, lymph nodes, spleen, tongue and blood of the test animals. Proviral DNA in 1 μg genomic DNA (equivalent to approximately 1.6×10^5 cells) was only detected in the blood cells of three SFVmac infected hamsters (data not shown). All other samples tested negative.

Discussion

Earlier studies had shown that rabbits and mice can be infected with various strains of foamy virus (Hooks and Detrick-Hooks, 1979; Saib et al., 1997; Swack and Hsiung, 1975). These animals were therefore used to establish animal models for artificial foamy virus infection. This publication presents a new small animal model using hamsters for the *in vivo* characterisation of foamy virus infection.

Our infection experiments included a comparison between the use of pure DNA to inoculate animals and the usual method of inoculating with infected cell culture supernatant. In our opinion, using a full-length clone for inoculation has a number of advantages, the major of which is the fact that a plasmid DNA preparation is virtually 100% free of cell culture components such as virus-opsonised cell-surface proteins, unclassified virus strains or mycoplasma. In addition, we could show that this route of application yielded a high rate of infection. Our preliminary experiments in which hamsters and mice were inoculated with PFV-DNA by gene gun also gave a 100% infection rate (10 out of 10, data not shown) whereas inoculation with virus-containing supernatant resulted in a number of failures to infect. The ELISA and western blot data indicate, in addition, that gene gun inoculation stimulated a different pattern of immune response in the hamsters. This may be due to the fact that the initial inoculating dose is very low compared to the i.p. inoculation with 10^6 particles. Following DNA inoculation, virus replication and release in transduced epithelial commences at a very low level. This is similar to the situation following natural infection because the initial transmission through biting or scratching is also likely to involve a few virus particles only. In addition, the storage and transport of gene gun ammunition is far simpler, a factor that could be important if recombinant replicating foamy viruses were to be used as vaccine vectors. However, the initial costs of purchasing gene gun equipment are, of course, relatively high.

It was possible to demonstrate the induction of a specific immune response in hamsters following inoculation with plasmid DNA coding for the PFV deletion U3-mutant HSRV2/13. Inoculation of mice in parallel allowed a direct comparison between the new hamster model and the established mouse model (Fig. 1). Using the newly established ELISA, specific antibodies appeared in hamsters at around 50 d.p.i. with a continuous increase in titre until day 92 and a partial drop and rebound in the last two bleedings. As this pattern was seen in all four infected hamsters, it might indicate a transient control of viraemia similar to that seen for other retroviruses such as equine infectious anaemia virus (Olsen, 1998), although this has so far not been shown for foamy viruses. To rule out the possibility that the immune responses observed were simply induced by DNA-immunisation via gene gun (Niederstadt et al., 2012; Siegismund et al., 2009), control experiments using a replication incompetent HSRV2/13 mutant were carried out. PFV-specific antibodies were not induced in any hamsters inoculated with this construct (supplement, Fig. 2.). Furthermore, the plasmids used the foamy virus LTR as promoter, which is activated exclusively by the spumavirus transactivator (Tas) (Erlwein and Rethwilm, 1993). A promoter-driven gene gun effect is therefore unlikely.

The pattern of antibody response was different in the mice, with four of five animals developing specific antibodies by 20 d.p.i, plateauing much higher than in the hamsters after 50 days and with no transient reduction in titre (shown in Fig. 1). This suggests a different course of foamy virus infection in the mice, with the drop in antibody response in all animals by the last bleeding suggesting a cessation of viral replication. It is possible that the strong immunological response to the infection cleared most of virus producing cells and the on-going virus release was either delayed or abrogated. Previous studies have demonstrated an immune response in BL/6 mice for as long as 24 weeks after infection (Schmidt et al., 1997b). This suggests a persistent and long lasting infection in mice. Whether or not the pattern of replication in the two species really does significantly and reproducibly differ should be clarified in further experiments.

In addition to the inoculation of full-length foamy virus genome by gene gun, a group of hamsters was also inoculated in parallel with infectious PFV cell supernatant. Although three of the four animals became seropositive by 20 d.p.i. with high antibody levels and a decrease in titre over time (compared to 45 days following DNA inoculation), one animal remained seronegative throughout the period of study, suggesting a failure to infect (shown in Fig. 1b). These findings could reflect the high number of virus particles in the initial inoculum. The strong immune response could result from a rapid establishment of high-level viral infection, a scenario not occurring during natural low level exposure. In this study, at least, PFV-DNA inoculation proved to be a more reliable method of infection.

Infection of cats with the feline foamy virus (FFV) is an additional animal model that has been characterised in detail (Alke et al., 2000; Schwantes et al., 2003). This system has the advantage of being based on a naturally occurring virus/host system in contrast to those based on the use of rodents. However, in contrast to the almost ubiquitous use of rodents, most laboratories do not have the capacity for housing cats, and an FFV/rodent model could be useful in these cases. A group of four Hamsters was therefore inoculated by gene gun with DNA (pCF-7, kindly provided from Martin Löchelt, DKFZ Heidelberg) coding for the feline foamy virus. A western blot with FFV-infected cell lysate showing the immune response 78 days after inoculation is provided in the supplement Fig. 1. Three of four animals show a double band around 50 kDa. We suggest this could be the FFV Gag double band p51/p48. However, a number of unspecific bands also appear in this blot. In contrast to the experiments using PFV-DNA, the FFV construct included the relatively strong CMV-EI promoter in place of part of the 5′-LTR (Schwantes et al., 2002), which means that an immune response to expressed proteins without infection, *i.e.* by 'DNA-immunisation' (Davis, 1997; Nguyen-Hoai et al., 2012; Witkowski et al., 2009), cannot be ruled out. However, the maintenance of the immune response for 165 days following inoculation indicates that the hamsters were indeed productively infected with FFV. An unequivocal verification of these results and determining whether or not the hamster model presented here provides a useful tool for evaluating FFV-based vectors will require additional studies.

In order to extend the range of foamy virus subtypes suitable for infection studies in hamsters, we carried out a series of infection studies using an SFVmac isolate. A number of studies have shown that circulating blood lymphocytes represent a major *in vivo* reservoir for foamy viruses and that the virus can be isolated from these cells by *in vitro* co-cultivation (Tobaly-Tapiero et al., 2005; von Laer et al., 1996). Cultivating one million PBMCs from an infected rhesus macaque with BHK-21 cells resulted in the successful recovery of a novel primary isolate of SFVmac. This wild-type strain was sequenced to facilitate classification and homology analyses. Due to the obvious differences to the published sequence, the new isolate was given the designation 'SFVmac-R289hybAGM', as it appears to have been formed by the recombination of two separate viral species. Recently, work has been published that demonstrates a similar recombination event an isolate of SFVmac deposited with ATCC (Galvin et al., 2013). This confirms our observation and makes it unlikely to be the result of a laboratory artefact. These findings also suggest a potential 'hotspot' for recombination events in the receptor binding domain of the foamy virus surface glycoprotein. This would certainly appear to warrant further investigation.

Hamsters and mice were inoculated intraperitoneally with infectious cell culture supernatant containing the new isolate. However, attempts to re-isolate virus or demonstrate proviral DNA by PCR only succeeded in the three hamsters infected with SFVmac-R289hybAGM. Lung tissue from all three of these animals and spleen cells from one animal yielded virus upon co-culture. In contrast, attempts to isolate virus from the organs of the SFVmac infected mice failed, indicating a lower level of virus replication or a stronger immune response in this species. This is also indicated by high antibody titres in the initial infection and the following drop in antibody titres over time to low levels.

It was also not possible to isolate virus from PFV-DNA inoculated animals (hamsters or mice), which is in agreement with previous studies (Schmidt et al., 1997b) in which re-isolation of PFV was only possible in 2 of 52 cases. Proviral DNA could only be detected by PCR in the PBMC of the three SFVmac infected hamsters, indicating that these cells are indeed a reservoir for foamy virus during this phase of infection, in agreement with observations in the natural host species (von Laer et al., 1996). Samples of saliva from none of the rodents infected here yielded infectious virus, which is in contrast to situation in naturally infected primates in which virus replication and release of viral particles has been demonstrated (Falcone et al., 1999a; Falcone et al., 2003). Based on the fact that we failed to detect viral DNA in the lung or spleen cells of SFVmac inoculated hamsters but could re-isolated virus from the same tissues, it would appear that re-isolation is the more sensitive method for detecting virus replication in animal tissues. As it is possible, in principle at least, to detect one infected cell in five million, a level of sensitivity very difficult to achieve by PCR, this is perhaps not surprising. The failure to re-isolate or detect PFV by PCR in all animal tissues may therefore reflect a genuinely very low level of replication *in vivo*. We did observe a replication advantage of SFVmac-R289hybAGM over PFV *in vitro* (data not shown), which could explain the differences in replication efficiency *in vivo*. However, the pattern

of antibody development strongly suggests that, despite the lack of detectable PFV in experimental animals, a productive infection via gene gun inoculation or supernatant infection had occurred. Specific antibodies were detectable over period of nearly 150 days and the increase in antibody titres between day 127 and 148 in both hamster groups (Fig. 1a and Fig. 2a) indicates an on-going viraemic phase of infection and persistent replication. The induction of neutralising antibodies nevertheless provides further evidence for a productive infection.

The development of FV-based vectors has clearly advanced in the recent years. However, evaluation of such vectors in primates, although ideal, poses many problems. In addition to the high costs both in terms of animals and infrastructure associated with primate studies, the availability of FV-negative animals is severely limited. Although the relevance for clinical use of immunological data obtained in small animal models is itself often questionable, these often provide an invaluable first step in the evaluation of such vectors. Our results demonstrate that, particularly for SFVmac, the replication in hamsters is clearly different from that in mice. The hamster model presented here therefore provides a further small animal system that facilitates a broader evaluation.

In summary, the small animal model presented here using the Syrian golden hamster introduces a relatively cost-effective, simple and reproducible tool for investigating foamy virus infection and recombinant vector systems *in vivo* that should be useful for studies in the future.

Methods

Foamy virus isolation and production of infectious cell culture supernatant

The primary isolate SFVmac-R289hybAGM was obtained by co-cultivating 2×10^6 PBMC from rhesus macaque 289 (Paul Ehrlich Institute) with BHK-21 cells in RPMI containing 10% FCS, 1% penicillin/streptomycin, 180 U/ml human IL-2 and 40 μg/ml PHA (Sigma Aldrich, Germany). After two weeks, the cells exhibited the cytopathic effect typical of foamy virus infection and cell-free supernatant was collected. DNA was isolated from the infected cells and the viral genome was amplified in sections by PCR for sequencing. Infectious cell culture supernatant for animal inoculation was obtained by transfecting BHK-21 cells with full-length genomic DNA of the HSRV2/13 strain of foamy virus (Schmidt et al., 1997a) and the SFVmac viral stock was obtained by infecting BHK-21 cells with the primary viral stock of SFVmac R298hybAGM. After two weeks the supernatants were filtered to remove cells, aliquoted and frozen in liquid nitrogen until use. Infectious titres were determined by endpoint dilution in BHK-21 cultures and detection of proviral DNA in cell lysates 72 hours later by real-time PCR.

Experimental infection of hamsters and mice with PFV/SFVmac by gene gun and by inoculation with infectious supernatant

Hamsters and mice were obtained from Charles River Inc. (Sulzfeld, Germany). Inoculation via gene gun was achieved using gold particles coated with the DNA. Briefly, viral DNA of the molecular clone pHSRV2/13 was coated onto gold particles (0.8–1.5 μm, Alfa Aeser GmbH & Co KG (Karlsruhe, Germany), according to the manufacturer's instructions using the Tubing Prep Station (Bio-Rad, Munich, Germany). The coated particles were then delivered to the upper epithelia of the experimental animal's shaved abdominal skin using the Helios Gene Gun (BioRad, Germany) driven by helium at a pressure of 300 psi. Each of the three shots at separate positions delivered approximately 1 μg DNA.

Infection with virus was achieved by intraperitoneal injection of 1 ml of cell-free culture supernatant containing 10^6/ml infectious PFV/SFVmac particles into anaesthetised animals (Isofluran). Blood samples were then taken at regular intervals and serum stored at -20 °C.

Organ preparation, isolation of genomic DNA and virus re-isolation

Samples of spleen, lung, tongue, liver, lymph node, intestine and blood were taken from the euthanised animals at the end of the experiment. PBMC were isolated by density gradient centrifugation of whole blood using Histopaque®-1077 (Sigma-Aldrich, Germany). Plasma was collected and stored at −20 °C. DNA was isolated from all organs by cell lysis, phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation according to standard procedures (Sambrook et al., 1989). The quantity and purity of the isolated DNA was determined photometrically at 260 nm and 280 nm. Single cell suspensions from the lungs and spleens were also prepared and co-cultivated (5×10^6 cells) with MRC-5 cells without stimulation. After five days, the primary cells were removed and the MRC-5 cells cultivated further for three weeks. Saliva samples were obtained from all animals by washing the oral cavity twice with 2 ml PBS. These were then centrifuged and cell-free supernatant added to MRC-5 cells together with 5 μg/ml Ciprofloxacin and also incubated for four weeks. Infection was detected by microscopic observation and by PCR. Virus re-isolation from hamster and mouse organs was achieved by co-cultivating single cell suspensions with MRC-5 cells in an equal mix of RPMI and MEM containing 10% FCS, 1% penicillin/streptomycin, 1 mM sodium pyruvate and 1 mM L-glutamine.

Immunoblotting and enzyme-linked immunosorbent assay (ELISA)

SDS-PAGE and semidry western blotting were performed using standard protocols with lysates of infected BHK-21 cells as antigen and sera diluted 1:750 to 1:1000 (hamsters), 1:250 (mice) or 1:1500 from a naturally infected rhesus macaque. ELISAs were also performed using standard protocols (Perkovic et al., 2010) with infected BHK-21 cell lysates to coat plates and sera diluted in three-fold steps from 1:20 to 1:43,740. Titres were calculated as the dilution at which the line of best fit crossed the cut-off OD (0.2).

Neutalisation assay

BHK21 cells were seeded into microtitre plates in 100 μl medium (DMEM-10% FCS) and allowed to attach and grow overnight. 20 μl of sera to be tested (undiluted or diluted 1:2 in medium) were mixed with 80 μl of virus-containing cell supernatant (5×10^4 infectious particles/ml) and incubated for 20 min at 37 °C. The mixtures were then added to the BHK21 cells (to give final dilutions of sera in the wells of 1:10 and 1:20). After a further 72 h incubation, supernatants were removed, the cells lysed and the levels of viral DNA measured by real time PCR (Behrendt et al., 2009). Neutralisation (NT) was expressed as n-fold reduction in viral DNA as calculated by: $NT = 2^{\Delta CT}$.

Real-time PCR-based detection of proviral DNA

A nested PCR using a generic foamy virus FAM-probe was established to facilitate detection of PFV and SFVmac-R289-hybAGM proviral DNA. The probe (5'-FAM-TTGGAATTCAGTAC-TCCTTATCACCC-BHQ1-3') allows most species of the foamy virus family to be detected as it binds to a highly conserved region of the *pol* gene. Appropriate primers flanking the probe were generated to allow specific detection of different subtypes. For PFV, the outer

primers giving a 482 bp product were 1Nes-F17-for (5′-GGTTA-TACCCCACTAAGGCTCCTTCTACTAGCGC -3′) and 1Nes-R18rev (5′-GGAACGAGAGGAGGCTGGAGGGGTGGATGG-3′) and the inner primers giving a 209 bp product were PFV-F1for (5′-CTTCAA-CCTTTGCTGAATG-3′) and PFV-R3rev (5′-TAATACAGGGCTATAGG-TGT-3′) (Mullers et al., 2011). For SFVmac-R289hybAGM the outer primers giving a 403 bp product were RH6-NesOut-for (5′-GGCTCCCTCAACTAGCGCAACTGTTAAAGCTCTC-3′) and RH6-NesOut2-rev (5′-GAGACAACTCCTCTTCTCTGGATAAATCAAGTGTAT-CAG-3′) and the inner primers giving a 257 bp product were RH6-NesIn-for (5′-CTACTTTTGCTGATTGGGC-3′) and RH6-NesIn-rev (5′-CAAACGGTGTGTTGGAATC-3′). The first reaction using 1 μg genomic DNA (approximately equivalent to 1.6×10^5 cells) as template was carried out with 95 °C/10 min pre-denaturation and 25 cycles of 95 °C/30 s to 67 °C/30 s to 72 °C/40 s. The second, nested real-time PCR using the generic FAM-probe was performed with 95 °C/10 min pre-denaturation followed by 50 cycles of 95 °C/30 s to 58 °C/30 s to 72 °C/20 s. Highly diluted genomic DNA from infected BHK-21 cells spiked with 1 μg genomic DNA from uninfected BKH-21 cells was used as positive control. Due to initial problems with DNA contamination encountered during the establishment of the nested PCR system, all organ preparations and PCRs were carried out under 'diagnostic' conditions. Dilutions of a plasmid standard indicated a detection limit of 5 copies per microgram DNA. We also developed a real time primer set for the feline foamy virus strain pCF-7 in the *pol* region named FFV-real-for (5′-CTGAAGAATTTGCTCAGTG-3′) and FFV-real-rev (5′-TACTTGGTGCTGACAACAT-3′). For this assay, the newly developed generic probe can also be used.

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Appendix A. supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.09.027.

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