Generation and characterization of genetic reassortants between Puumala and Prospect Hill hantavirus in vitro

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Hantaviruses belong to the family Bunyaviridae characterized by tri-segmented RNA genomes. Depending on the hantavirus species, infection can lead to hantavirus cardiopulmonary or haemorrhagic fever with renal syndrome. In vitro studies suggest that pathogenic hantaviruses evade induction of innate antiviral responses, and this ability might determine the virulence in humans. Since reverse genetic systems are not available, in vitro reassortment is currently the only way to culture defined hantavirus variants. Here, we demonstrate for the first time the generation of a reassortant between a pathogenic Old World and a non-pathogenic New World hantavirus in vitro. The reassortant contained the glycoprotein coding M-segment derived from the pathogenic Puumala virus (PUUV) and the other genomic segments coding for the nucleocapsid protein and RNA-dependent RNA-polymerase from Prospect Hill virus (PHV), which is taken as non-pathogenic in humans. Exchange of the M-segment was confirmed by sequencing and virus neutralization test with PUUV-specific sera. Functional analysis of the reassortant and parental viruses revealed characteristic growth kinetics and innate immune responses as determined by expression analyses for lambda interferon and MxA, and by interferon-stimulated response element reporter gene studies. Consistent with previous studies with other pathogenic hantaviruses, PUUV elicited reduced innate responses if compared with PHV. In all these functional assays the reassortant revealed PHV-like phenotypes. Thus, neither the PUUV M-segment nor entry via specific M-segment directed pathways modulated the virus type-specific innate responses. Moreover, the data imply that this approach might be an option for production of attenuated viruses that could be used as vaccines against pathogenic hantaviruses.

INTRODUCTION

Members of the genus Hantavirus in the family Bunyaviridae are characterized by an enveloped virion with a segmented genome consisting of three single-stranded RNA molecules. The genomic segments designated L- (large), M- (medium) and S- (small) RNA code for an RNA-dependent RNA-polymerase, two glycoproteins G1/G2 and a nucleocapsid protein (N-protein), respectively (Elliott et al., 1991). Hantaviruses exhibit narrow host range specificity as each virus species is found almost exclusively in one or few rodent/insectivore host species. In these small animals, the virus persists usually without obvious symptoms. Transmitted to humans, hantaviruses can lead to hantavirus cardiopulmonary syndrome (HCPS) or haemorrhagic fever with renal syndrome (HFRS), depending on the virus species involved. HCPS caused by hantaviruses found in the Americas like Sin Nombre virus (SNV), New York virus (NYV) or Andes virus (ANDV) is associated with case fatality ratios of up to 40%. HFRS caused by Old World hantaviruses like Hantaan virus (HTNV) or Dobrava Belgrade virus (DOBV) exhibits fatality rates of up to 14%. In Northern and Central Europe, Puumala hantavirus (PUUV) is found to cause nephropathia epidemica, a mild HFRS variant with a fatality index below 0.2% (Krüger et al., 2001; Schmaljohn & Hjelle, 1997; Schönrich et al., 2008). In addition to the pathogenic hantaviruses, several members of the genus can be considered to be non-pathogenic, i.e. Prospect Hill virus (PHV) has never been reported to cause disease in humans, despite the widespread distribution of PHV in indigenous rodents (Yanagihara et al., 1987). The phylogenetic relationship between selected hantavirus species is presented in Fig. 1.

The molecular mechanisms for species-specific virulence of hantaviruses in humans are not resolved. Analysis of the pathogenicity in vivo is hampered by the fact that suitable experimental animal systems are only available for a few
hantavirus species (Hooper et al., 2001; Klingström et al., 2008). In vitro, two phenotypic attributes have been identified that correlate with the pathogenicity of hantaviruses in vivo. First, pathogenic hantaviruses including NYV, HTNV and PUUV were reported to enter host cells via integrin-αv/β3. Hantaviruses considered to be non-pathogenic, like PHV, were found to enter the cell via integrin-αII/β1 (Gavrilovskaya et al., 1998; Mackow & Gavrilovskaya, 2001). Second, several pathogenic hantaviruses elicit delayed innate antiviral responses when compared with an immediate response triggered by non-pathogenic hantaviruses (Alff et al., 2006, 2008; Geimonen et al., 2002; Handke et al., 2009b; Khaiboullina et al., 2004; Kraus et al., 2004; Spiropoulou et al., 2007). Tumour necrosis factor receptor-associated factor 3 (TRAF3) was indicated to be crucial for innate antiviral responses both to pathogenic and non-pathogenic hantaviruses (Handke et al., 2009b). In contrast to this common feature, the NY-1 hantavirus G1 cytoplasmic tail, but not the corresponding region of PHV, blocked activation of IRF3 by destabilization of the TBK1/TRAF3 kinase adaptor complex (Alff et al., 2006, 2008). Moreover, it was shown that the Toll-like receptor 3 (TLR3) is involved in innate responses triggered by HTNV late after infection, which was not required for the induction of antiviral genes by PHV (Handke et al., 2009b). These data indicate that specific antagonistic activities and/or selected recruitment of different pathogen recognition receptors might be decisive for the differential antiviral responses to infection with pathogenic and non-pathogenic hantaviruses.

Genetic reassortment is known to play an important role in the evolution of segmented RNA viruses. Generation of reassortants between different hantavirus strains has been reported by several groups in vivo and in vitro. In most of the cases the described reassortants derived from different lineages of the same hantavirus species (Henderson et al., 1995; Klempa et al., 2003; Li et al., 1995; Razzauti et al., 2009; Rizvanov et al., 2004; Rodriguez et al., 1998; Zou et al., 2008). Between different virus species reassortment is restricted in vivo due to the narrow host range, which prevents dual infection of the same host and cell. In addition to this primary limitation, in vitro studies revealed that further viral factors repress reassortment between unrelated hantaviruses. At least for some species, this repression can be overcome, as documented by the isolation of stable reassortants produced after dual infection of Vero (African green monkey epithelial kidney cells) cells with SNV and ANDV or Black Creek Canal virus (BCCV) (McElroy et al., 2004; Rodriguez et al., 1998).

In this study, we generated reassortants between the pathogenic PUUV and the non-pathogenic PHV. The isolated reassortants (PHPUV) contained the M-segment derived from PUUV and the S- and L-segments from PHV. In order to characterize the reassortants, growth kinetics and induction of innate antiviral responses were compared to the characteristic phenotypes of the parental viruses.

RESULTS

Generation of reassortants between PHV and PUUV

Initially, 54 clones were processed via focus purification (Rang et al., 2006), in order to isolate reassortant viruses generated by co-infection of Vero cells with PUUV and PHV. Genotyping of the obtained clones was performed by virus species- and segment-specific PCR. By these means one clone was indentified that contained a diploid M-segment derived from PUUV and PHV and the S- and L-segments from PHV. All other clones exclusively contained segments derived from the parental PHV. The diploid clone was subjected to an additional focus purification cycle and 23 secondary clones were isolated and analysed. By PCR-genotyping, 13 clones were found with the same diploid M-segment genotype as the origin input virus. Three clones contained genomic RNA that were all derived from PHV and seven haploid reassortant clones contained the S- and L-segments from PHV and the M-segment from PUUV. Genotyping of three reassortant clones via PCR is presented in Fig. 2.

All further experiments were performed with clones #2 and #3. The genomic reassembly pattern of these clones was confirmed by partial sequencing of each of the three segments. The genomes of the reassortant virus clones and
of the parental PHV and PUUV were amplified by RT-PCR with the primer pairs PHV S150–S1350, PUUV M1–M3682 and PHV L4910–L6559 (primer information is shown in Supplementary Table S1, available in JGV Online). Both strands of the PCR amplicons derived from the L- and S-segments of the reassortant and parental viruses were determined. Except for the 314 bp region from nucleotide positions 1715–2029, according to the GenBank accession number NC_005223, the M-segment of the reassortant and the parental virus was sequenced completely. Alignment of the resulting data confirmed the reassortment patterns determined by species- and segment-specific PCR. Furthermore, sequence analysis did not reveal any nucleotide exchange between the reassortant and the parental genome (data not shown). These results demonstrate in vitro production of a reassortant between the pathogenic PUUV and non-pathogenic PHV. The generated reassortant was designated PHPUV. Stocks produced with the PHPUV clones #2, #3 and #6 derived from a single M-segment diploid clone, which was passaged once and used for an additional focus purification cycle. Neg, PCR control without template.

of virus-neutralizing titres represents one criterion for hantavirus species definition (Fauquet et al., 2005). Against PHPUV the sera revealed a similar reactivity (1/640) as observed with PUUV (Table 1). The latter finding with PHPUV, which contains the PUUV-encoded glycoproteins, confirms the genotypic characterization.

Replication of the reassortant PHPUV and parental viruses PHV and PUUV

Next, we determined replication of the reassortant PHPUV compared to the parental hantaviruses PHV and PUUV in type I interferon (IFN)-deficient Vero cells (Diaz et al., 1988) and in IFN-competent A549 (human epithelial lung cells) and HuH7 (human hepatoma cells) cells. In Vero cells, PHPUV and the parental viruses grew with similar efficiency, yielding titres of $1 \times 10^5$ to $1 \times 10^6$ f.f.u ml$^{-1}$ (Fig. 3). In IFN-competent A549 cells replication of all viruses was also similar, albeit the amount of virus produced was about 3 logs lower compared with the yield in Vero cells. Interestingly, PUUV replicated poorly not

**Table 1.** Reactivity of sera from convalescent PUUV-infected patients against PUUV, PHV and PHPUV in FRNT

<table>
<thead>
<tr>
<th>Virus</th>
<th>FRNT end-point titre</th>
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<tr>
<td>Serum A</td>
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<tr>
<td>PUUV</td>
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<td>PHV</td>
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<td>PHPUV</td>
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**Fig. 2.** Genotyping of isolated reassortants by species- and segment-specific PCR. Virions secreted into the cell culture medium 5 days after co-infection of Vero cells with PHV and PUUV were harvested and cloned via focus purification as described previously (Rang et al., 2006). Specificity of the primer pairs used is indicated. Reassortant clones designated #2, #3 and #6 derived from a single M-segment diploid clone, which was passaged once and used for an additional focus purification cycle. Neg, PCR control without template.

**PHPUV reacts like PUUV in focus reduction neutralization tests (FRNT)**

Initially, the reactivity of two sera from convalescent PUUV-infected patients against PUUV, PHV and PHPUV was tested by FRNT. In dilutions higher than 640-fold both sera neutralized PUUV infectivity below the cut off. The sera had to be used in 160- and 340-fold dilution to show a similar neutralization against PHV. This result confirms a well documented cross-reactivity between different hantavirus species, which are closely related to PHV and PUUV (Chu et al., 1995; Lee et al., 1985; Lundkvist et al., 1991; see Fig. 1). Despite this cross-reactivity, the reciprocal neutralizing end-point titre against PUUV was at least fourfold higher compared with PHV. A fourfold difference

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only in A549 but also in HuH7 cells, in which both PHPUV and PHV replicated reproducibly to more than 40-fold higher titres. Thus, PHV and PUUV revealed a distinct replication pattern in HuH7 cells. Interestingly, PHPUV replicated with the same characteristics in all cell lines, including HuH7, as PHV. Although, it is unclear why PUUV replicated less efficiently in HuH7 cells compared with PHV and PHPUV, the data indicate that the M-segment is not decisive for this phenotypic difference.

**PHV and PHPUV, but not PUUV, elicit similar characteristic innate antiviral responses**

To monitor innate responses to the infection with PHV, PUUV and PHPUV mRNA expression of type I IFN-β, type III IFN-λ1, and of the type I and type III IFN-inducible MxA was analysed. Virus-mediated induction of IFN-β mRNA was not detected in A549 or HuH7 cells (data not shown). In contrast to type I IFN, in A549 cells PHV elicited high mRNA levels of IFN-λ1 and IFN-λ2/3, which steadily increased during the examined time frame (Fig. 4). Also in A549 cells, PUUV-infection induced IFN-λ mRNA, but the levels were up to 3 logs lower compared with PHV. As in A549, in HuH7 cells a similar differential IFN-λ response was observed. However, the IFN-λ mRNA levels were at least 10-fold lower in HuH7 compared with A549 cells.

A characteristic expression of MxA mRNA was found in PHV- and PUUV-infected cells. Compared with PUUV, at least 10-fold higher MxA mRNA levels were induced by PHV in both cell lines. Interestingly, the kinetics of the MxA mRNA expression did not correlate with the induced mRNA levels of IFN-λ. Already at 6 h post-infection (p.i.), maximal levels of MxA mRNA were found, which remained upregulated to similar levels in A549 and HuH7 cells within the experimental time frame.

Next, expression of MxA on the protein level was analysed. Cells were harvested at indicated days p.i. and expression of MxA and viral N-protein were determined by Western blot analysis (Fig. 5). Consistent with the data published previously, PHV triggered early expression of MxA in both...
A549 and HuH7 cells. In contrast to this pronounced response to PHV, in A549 cells infected with PUUV no MxA induction was found. Productive infection of these cells with PUUV is indicated by increasing amounts of N-protein produced during the observation period. Furthermore, PUUV and PHV replicated to similar titres in A549 cells. Thus, reduced infectivity of A549 cells by PUUV compared with PHV can hardly explain differential MxA induction.

Despite this anergy in A549 cells and primary endothelial cells (data not shown), in HuH7 cells PUUV elicited an early but transient MxA protein expression pattern. MxA protein was clearly detectable at 1 day p.i., but its amount decreased over the next few days and declined below the detection level by day 4. Because the amount of MxA mRNA did not decline, this transient protein expression pattern seems to be regulated post-transcriptionally. As in A549, in HuH7 cells increasing amounts of PUUV N-protein and similar PUUV titres were produced. In contrast to studies performed with other hantaviruses in these and other cell systems, expression of MxA did not inversely correlate with the amount of infectious PUUV produced (Alff et al., 2006; Handke et al., 2009b).

Although the function and significance of these findings are unclear, the presented data demonstrate that PHV and PUUV induce ISRE activation in a dose-dependent manner (Fig. 6). In cells infected with PHV and PHPUV with an m.o.i. of 1 the ISRE-directed expression was 20- and 30-fold higher, respectively, compared with the uninfected control. Like PHV and PHPUV, PUUV also activated the transfected ISRE, however, the level was about four to fivefold lower. Consistent with the results presented above these results demonstrate that PHV and PUUV differenti-
ally induce innate antiviral immune responses in vitro. In all experiments PHPUV revealed the same characteristic innate antiviral response pattern as PHV, which can be considered to be a non-pathogenic hantavirus.

**DISCUSSION**

Here, we demonstrate for the first time successful generation and isolation of reassortants between a pathogenic and non-pathogenic and between an Old World and a New World hantavirus. To the best of our knowledge so far only three stable reassortant clones have been produced and isolated in vitro, which were derived from two different pathogenic hantavirus species (McElroy et al., 2004; Rodriguez et al., 1998). Two of these reassortants were isolated after dual infection of Vero cells with SNV and ANDV and screening of 208 individual clones. One reassortant was found in 163 clones isolated after coinfection with SNV and BCCV. In all these cases, as in our study, exclusively the M-segment was exchanged. These findings suggest that assortment is controlled by species-specific determinants, which most tightly prevent segregation of the homologous S- and L-segments. The data clearly demonstrate that restricted heterologous reassortment can be overcome at least for the M-segment. The requirements and restrictions for both homologous assortment and heterologous reassortment are unknown. After dual infection of Vero cells with DOBV strain Slovakia and DOBV strain Slovenia, only M-segment reassortants (65 clones) were isolated, beside clones with the parental (72 clones) and unstable diploid genomes (65 clones) (Kirsanovs and others, unpublished). In vivo a similar preference for the M-segment reassortment was reported for SNV (Henderson et al., 1995; Li et al., 1995). In contrast to the reassortment patterns observed for SNV and DOBV, no M-segment preference was found in mice infected with two different PUUV lineages (Razzauti et al., 2009). In the latter study S-, M- and L-segment reassortants were identified. Thus, assortment might be directed at least in part by different species-specific determinants that restrict reassortment. Based on the data currently available, it is not possible to predict the limitations and options of this in vitro approach, and whether reassortants can also be produced with hantaviruses as distantly related as HTNV and PHV.

As described recently for HTNV and PHV (Handke et al., 2009b), we compared the phenotype, i.e. replication and induction of innate responses, of the reassortant and the parental viruses in different cell lines. Consistent with the response triggered by HTNV, PUUV infection did lead to reduced innate responses compared with PHV as determined by quantification of the mRNA of type III IFN and MxA and reporter gene experiments. Despite this reduced responsiveness, PUUV replicated in A549 cells as poorly as PHV, which induced a pronounced antiviral response. In HuH7 cells, PHV replicated to higher titres than in A549 cells. This increase in replication was not found in PUUV-infected HuH7 cells. Based on these data, it seems obvious that the measured antiviral responses induced by PUUV, which are clearly not as pronounced as the responses triggered by PHV, can hardly explain poor replication of PUUV in A549 and HuH7 cells. Recombinant IFN-α blocked PUUV and HTNV with a similar effective concentration in Vero cells (data not shown). The latter finding suggests that increased sensitivity of PUUV against low level antiviral responses as measured in PUUV-infected HuH7 cells seems not to be the decisive reason for poor PUUV titres in this cell system. Therefore, we assume that restricted replication of PUUV in these IFN-competent cells might be limited by inherent viral or cellular mechanisms independent from innate antiviral responses.

Differential induction of innate responses observed in primary endothelial cells was suggested to be mediated via IFN-β (Alff et al., 2006; Kraus et al., 2004). In our experimental system we could not detect induction of IFN-β mRNA. In contrast to type I IFN, we demonstrate for the first time induction of type III IFN by hantaviruses. Moreover, our data demonstrate that PUUV and PHV differentially induced expression of IFN-λ mRNA in our experimental system. These results suggest that in addition to type I IFN type III IFN might play an important role for the induction of differential innate antiviral responses elicited by pathogenic and non-pathogenic hantaviruses. Kinetic mRNA analysis revealed that expression of IFN-λ and MxA did not match, at least in the early phase of postinfection. Currently it is not clear, whether minor amounts of type I IFN might be present and contribute to the induction of IFN-stimulated genes. Further studies are required to address this interesting question.

Moreover, the expression pattern of type III IFN mRNA cannot explain transient expression of the MxA protein in PUUV-infected HuH7 cells. Quantification of the mRNA suggests that turn-over of the MxA protein seems to be modulated by PUUV on the post-transcriptional level. Interestingly, almost the same MxA protein and mRNA expression patterns were found in HuH7 cells infected with DOBV strain Slovenia (Kirsanovs and others, unpublished). This characteristic MxA expression pattern might suggest that PUUV and DOBV strain Slovenia can directly antagonize antiviral responses. Although the human hepatoma cell line HuH7 does not mirror the primary target cell type infected by hantaviruses in vivo, this observation and experimental system might help to reveal principle mechanisms of the interaction between hantaviruses and the innate immune system.

Functional characterization of the reassortant PHPUV revealed the same replication and antiviral response patterns as observed for the parental PHV, which were clearly distinct from PUUV. These results indicate that none of these characteristic features were determined by the M-segment. The data clearly indicate that the S- and L-segments of PHV and PUUV seem to be decisive for the
differential induction of innate responses and replication efficiency in the experimental system used.

PUUV and PHV have been shown to enter host cells via different integrin receptors (Gavrilovskaya et al., 1998; Mackow & Gavrilovskaya, 2001). Since entry of hantaviruses is determined by the viral glycoproteins G1/G2, the M-segment should direct the reassortant PHPUV to the same uptake pathway that is also used by the parental PUUV. PHV and PHPUV triggered an early and pronounced antiviral response in A549 and primary endothelial cells (data not shown), irrespective of which entry pathway was used. Hence, the data imply that different receptor interactions and subsequent entry pathways displayed by PHV and PUUV might not be decisive for the differential innate responses induced.

Consistent with studies for HTNV, high-level replication of the pathogenic NYV in primary endothelial cells correlated with a reduced innate antiviral response early after infection (Alff et al., 2004). The pathogenic NY-1 hantavirus G1 cytoplasmic tail has been shown to block activation of IRF3 by destabilization of the TBK1/TRAF3 kinase/adaptor complex (Alff et al., 2006, 2008). It is unclear whether this antagonistic activity is also conserved in other pathogenic hantaviruses and decisive for successful replication and dissemination in vivo. Our data indicate that at least the PUUV G1 cytoplasmic tail was not sufficient to block recruitment of TRAF3 in A549 cells, which has been shown to be involved in PHV-mediated induction of innate responses (Handke et al., 2009). Recently, it has been shown that PUUV can express a non-structural protein (NSs), which revealed a weak antagonistic activity against induction of type I IFN (Jääskeläinen et al., 2007). Although it is unclear whether the limited antagonistic activity described for the NSs-protein in vitro can account for the reduced responsiveness observed in PUUV-infected A549 cells and primary endothelial cells (data not shown), these reports suggest that independent antagonistic mechanisms might have evolved during co-evolution of hantaviruses within their specific hosts.

Retarded induction of innate responses in primary endothelial cells seems to be a conserved feature of several pathogenic hantaviruses including HTNV, SNV, ANDV, DOBV Slovakia, DOBV Slovenia and PUUV (Alff et al., 2006, 2008; Geimonen et al., 2002; Handke et al., 2009b; Khaiboullina et al., 2004; Kirsanovs and others, unpublished; Kraus et al., 2004; Spiropoulou et al., 2007). Despite this common feature, it has to be mentioned that PUUV replicated very poorly in IFN-competent cells in striking contrast to other pathogenic hantaviruses including HTNV and DOBV (Handke et al., 2009b; data not shown). This difference might reflect the fact that PUUV generally causes a mild form of HFRS and represents a virus with reduced virulence as compared with the other pathogenic hantaviruses.

In all functional studies, except for the FRNT, the reassortant PHPUV revealed the same characteristic phenotype as PHV in our experimental system. Compared with the non-pathogenic, all pathogenic hantaviruses tested consistently induced innate antiviral responses in a retarded manner in A549 and primary endothelial cells. If efficient induction of immediate innate responses represents the major characteristic feature for non-pathogenic hantaviruses, the reassortant PHPUV might be as non-pathogenic as PHV. Our data demonstrate that the antibodies elicited in PUUV-infected patients efficiently neutralize infectivity of the reassortant PHPUV. Vice versa PHPUV infection should elicit neutralizing antibodies also against the pathogenic PUUV and might be considered as attenuated vaccine. Further studies, i.e. infection experiments in macaques, are required to estimate the virulence of PHPUV and the role of the PUUV M-segment in the context of the other segments derived from PHV in vivo.

Although many research efforts have been tried, there is no WHO-approved vaccine available against hantavirus infections until now (Schmaljohn, 2009; Ulrich et al., 2002). Currently, formalin-inactivated HTNV is used as a vaccine in South Korea. However, the protective response to this vaccine is limited and requires continuous vaccination every second year to gain sufficient protection (Cho & Howard, 1999; Cho et al., 2002; Sohn et al., 2001). Reassortants between HTNV and PHV, which present the immunogenic properties of HTNV with the non-pathogenic properties of PHV, might be a promising option for the development of a novel vaccination strategy.

**METHODS**

**Cell culture and virus.** Vero, A549 and HuH7 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco Invitrogen) supplemented with 10% fetal calf serum (ThermoScientific), 100 IU penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ (both Gibco Invitrogen).

Virus stocks were produced in Vero cells derived from PUUV strain Sotkamo and PH strain 3571 that had been focus purified as described previously (Rang et al., 2006). Virus stocks were harvested 7 days p.i. For the experiments, stocks with titres with at least 8 × 10⁷ f.f.u. ml⁻¹ were used. Cells were infected with the indicated m.o.i. After 1 h at 37 °C, cell culture medium was added to the infectious inoculum. In experiments aimed to determine the replication kinetics of the viruses, cells were washed three times with PBS to remove input virus not bound to the cells. All virus stocks and cells were determined to be free of mycoplasma contamination as determined by PCR analysis.

**Determination of virus titres.** The amount of infectious virus secreted into the supernatant of infected cells was titrated in Vero cells (Handke et al., 2009a). Cells grown in 24-well plates were infected by different dilutions of the harvested supernatants for 1 h at 37 °C, fed with medium containing 0.5% agarose and incubated for 10 days at 37 °C. Thereafter, the medium/agarose overlay was removed and cells were washed with PBS and fixed with methanol for 10 min. Virus-infected cell foci were detected using a polyclonal rabbit serum. The formed antigen–antibody complexes were visualized using chemiluminescence super signal west dura according to the protocol supplied by the manufacturer (Pierce). The number of antigen-positive foci were counted and according to the dilution applied, the corresponding virus titre was determined as f.f.u. ml⁻¹.
Generation of reassortants between PUUV and PHV. Vero cells were infected with PUUV and PHV each with an m.o.i. of 0.1. After incubation for 1 h at 37 °C, the cells were washed three times with PBS to remove input virus not attached to the cells. Five days later, progeny virus secreted into the cell culture medium was harvested and the titre was determined as described above. For isolation of potential reassortants about 50 virus clones were isolated by three cycles of focus purification, as described previously (Rang et al., 2006). Vero cells grown in six-well plates were infected with the harvested virus stock in dilutions that should give one to three single virus clones per well. After infection, the cells were fed with medium containing 0.5% agarose and incubated for 10 days at 37 °C. Before removal of the medium/agarose overlay, the culture plates were incubated at 4 °C for 15–30 min to allow convenient handling of the overlay. Thereafter, the overlay was engraved at position 6 o’clock to mark the orientation, lifted off from the cell layer, and stored at 4 °C as the source for subsequent recovery of single virus clones. Cells were washed with PBS, fixed with methanol for 10 min and virus-infected cell foci were detected as described above. The image of the signals determined via immunodetection, which represents the infected cell foci, was mirrored horizontally using the Adobe Photoshop 7.0 software (Adobe Systems), because the overlay was turned accordingly when it was removed from the cells. A hard copy of this processed image was held under the agarose overlay in a Petri dish and used as a map to trace and pick the virions diffused into the corresponding region of the overlay. The picked material was mixed vigorously in 200 μl medium and used for reinfection of Vero cells.

Viral RNA isolation and screening for reassortants. Viral RNA was isolated from cell culture medium using the QIAamp Viral RNA Mini kit according to the protocol supplied by the manufacturer (Qiagen). Complementary DNA (cDNA) was transcribed from 10% of the isolated RNA using random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen) for 90 min at 37 °C following the recommendations of the manufacturer. For PCR, 2 μl of a tenfold dilution of the cDNA was used as template with 1 U Taq DNA Polymerase (Rapidozym) in 10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, with 200 μM each dNTP and 0.2 μM of forward and reverse primers in a final volume of 25 μl. PCR conditions were 40 cycles of 1 min at 94 °C, 1 min annealing (Supplementary Table S1) and 1 min at 72 °C, with 10 min at 95 °C prior to the reaction and 10 min of final extension at 72 °C in the T-personal 48 cycler (Biometra). The sequences and annealing temperatures of the corresponding primer pairs are shown in Supplementary Table S1.

Sequencing of the parental and the reassortant virus. RNA was isolated from virions secreted into the cell culture medium as described above. RT-PCR was performed with the Transcriptor High Fidelity cDNA Synthesis kit and the Expand Long Template PCR System with random hexamer primers according to the supplied protocols (Roche). PCR products were purified with the QIAquick PCR Purification kit (Qiagen) and sequenced. Primers used for amplification and sequencing are presented in Supplementary Table S1.

FRNT. FRNT was performed as described previously (Heider et al., 2001). Briefly, serial dilutions of convalescent sera from PUUV-infected patients were incubated for 1 h at 37 °C with 50 f.f.u. PUUV, PHV or PHPUV. Thereafter, virus–serum mixtures were added to confluent Vero cells and incubated for 1 h at 37 °C. Cells were fed with medium containing 0.5% agarose and incubated for 10 days at 37 °C. Infectivity of the samples was quantified as described above. The titre of neutralizing antibodies reflects the reciprocal dilution of the serum sufficient to reduce infectivity by more than fivefold, compared with the virus control without serum.

Total RNA isolation and quantitative RT-PCR analysis. Total RNA was isolated using the High Pure RNA Isolation kit (Roche) according to the manual supplied. cDNA was produced as described above from 2 μg total RNA. Five per cent of cDNA produced was used as template for real-time PCR in a final volume of 20 μl using LightCycler Fast Start DNA Master SYBR Green I kit (Roche) or Platinum SYBR Green qPCR SuperMix-UDG plus 5 μg BSA (Invitrogen) in a LightCycler 1.2 (Roche). Nucleotide sequences of the primers used for real-time PCR are shown in Supplementary Table S1. To avoid unintended amplification of genomic DNA, which might be present in the RNA preparation, intron spanning primers were used. Due to sequence similarities of the IFN-α2 and IFN-β3 mRNAs both transcripts were detected together with the same primer pair. The amount of RNA was standardized with the amount of GAPDH, which served as an invariant internal control for reverse transcription and quantitative PCR.

Western blot analysis. Protein extracts were prepared as described previously (Handke et al., 2009b). Extracts were separated by 10% SDS-PAGE and blotted onto nitrocellulose membrane (Whatman). Signals were visualized by enhanced chemiluminescence (Pierce).

Reporter gene assay. HuH7 cells grown in 24-well plates were transfected with 500 ng of an ISRE-controlled luciferase expression plasmid (pISRE-Luc; Clontech Laboratories #631913) using Lipofectamine 2000 (Invitrogen) according to the supplied protocol. The reporter gene construct contains five copies of a consensus sequence that can be bound (Tanaka et al., 1993) and activated by IRF1 in response to type I and type III IFN (data not shown). Six hours post-transfection, cells were infected with indicated virus and m.o.i. One day later, cellular extracts were harvested and the luciferase activity was quantified using a luciferase detection kit (Roche). For unknown reasons stimulation of ISRE-controlled luciferase expression was not detectable in A549 cells neither by infection nor addition of recombinant IFN-α (data not shown).

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