Infection of in vivo differentiated human mast cells with hantaviruses

Sven Guhl, Renate Franke, Anika Schielke, Reimad Johne, Detlev H. Krüger, Magda Babina and Andreas Rang

Increased vascular permeability is a key feature of the pathological symptoms caused by hantaviruses. Here, we analysed the interaction between hantaviruses and mast cells, which regulate vascular homeostasis. In highly purified human skin mast cells increasing amounts of Hantaan (HTNV) and, to a lower extent, Prospect Hill (PHV) virions were produced. Replication was confirmed by the production of viral plus-strand RNA as determined by a virus strand-specific RT-PCR. PHV but not HTNV elicited early expression of beta interferon, MxA, ISG15 and CCL5 consistent to studies with other cell types. The data demonstrate that mature mast cells are permissive to infection with hantaviruses. This interaction might contribute to the development of vascular leakage syndrome.

Hantaviruses (family Bunyaviridae) are characterized by a tripartite single-stranded RNA genome of negative polarity within an enveloped virion. The natural hosts are mainly rodents, in which the virus persists without obvious pathogenic symptoms. Transmission to humans via the respiratory tract can cause haemorrhagic fever with renal syndrome (HFRS) or hantavirus cardiopulmonary syndrome (HCPS) with case fatality rates up to 50%, depending on the hantavirus species involved. HCPS caused by New World hantaviruses leads to progressive pulmonary oedema, respiratory insufficiency and myocardial depression. HFRS caused by Old World hantaviruses generally is associated with retroperitoneal oedema and can pass through a febrile, hypotensive, oliguric and diuretic phase. Increased vascular permeability and thrombocytopenia are key features in both HCPS- and HFRS-patients and almost all severe cases are complicated by shock (Khaiboullina et al., 2005; Peters et al., 1999; Schmaljohn & Hjelle, 1997; Schönrich et al., 2008). The mechanisms and viral determinants responsible for the pathogenesis are unclear.

Hantaviruses can infect different cell types like endothelial, epithelial and dendritic cells (Raftery et al., 2002; Zaki et al., 1996). In these cells cytopathic effects have not been observed. However, subtle modulation of cell motility and permeability of primary endothelial cells and cell layers, respectively, by hantaviruses has been described previously (Gavrilovskaya et al., 2002, 2008). Furthermore, infection was associated with increased levels of inflammatory cytokines and chemokines including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β and CCL5 in cell culture as well as in infected patients (Geimonen et al., 2002; Linderholm et al., 1996; Sundstrom et al., 2001; Temonen et al., 1996). Based on these and other observations, direct modulation of endothelial cell function and/or immunopathological responses to the infection are currently thought to cause HCPS and HFRS (Khaiboullina et al., 2005; Peters et al., 1999; Schmaljohn & Hjelle, 1997; Schönrich et al., 2008).

Mast cells (MC) can modulate vascular homeostasis and play a crucial role in allergy and anaphylaxis, but it has become increasingly clear that the physiological relevance of MC lies in the regulation of innate and acquired immune responses. MC originate from haematopoietic progenitor cells, but undergo terminal differentiation exclusively in tissues in which they become ultimately resident tissue MC (Kirshenbaum et al., 1999). MC maturation occurs in close contact with the surrounding tissue and only MC, which have undergone tissue-dependent maturation can therefore be regarded as natural, in situ differentiated MC. Mature MC reside in mucosal and connective tissues in close proximity to the endothelial cell layer of blood vessels and are most prominent in those regions close to the external environment like the skin and mucosa of the lung and digestive tracts. This localization and expression of several toll-like receptors by human MC coincides with their function as immune sentinels that participate in defence against pathogens, such as nematodes, bacteria and certain fungi (Galli et al., 2005; Heib et al., 2008; Knellinger & Rocken, 2009; Kulka & Metcalfe, 2006; Marshall, 2004; Mekori & Metcalfe, 2000).

MC produce an extensive array of different vasoactive and inflammatory cytokines, chemokines, bioactive amines,
proteases and lipid-derived mediators. High amounts of these products can be stored in intracellular granules or rapidly synthesized, e.g. VEGF, TNF-α and CCL5. Depending on the stimulus and mediator type these compounds are released either by degranulation or secretion (Okumura et al., 2003; Varadarajalu et al., 2003). Interestingly, significantly increased levels of histamine were found in HFRS patients, pointing towards MC activation in the course of the infection (Sidelnikov & Sivoraksha, 1990, 1996).

Numerous studies have shown that effective immune responses towards different microbial pathogens require the presence of MC (Galli et al., 2005). However, while the concept of MC in mediating productive immune responses against bacterial infections is now broadly accepted, data on the role of MC in viral infections and their infectability by viruses are still fairly limited. MC have been proposed to serve as a latent reservoir for human immunodeficiency virus, since immature circulating MC precursors are susceptible to the virus, although this ability is lost rapidly as they mature towards tissue MC (Bannert et al., 2001; Sundstrom et al., 2007, 2009). Furthermore, it has been shown that dengue virus can infect MC/basophile-like cell lines and cord blood-derived MC (Brown et al., 2006; King et al., 2000, 2002). In these in vitro systems dengue virus infection was associated with increased release of inflammatory mediators, e.g. IL-1β and CCL5 (King et al., 2002). Therefore, MC might be involved in the development of thrombocytopenia and increased vascular permeability, characteristic for dengue virus-mediated haemorrhagic fever and dengue virus shock syndrome (Tan & Alonso, 2009).

Because of the prominent role for vascular permeability and shock, conditions in which the role of MC is highly appreciated, we studied whether in vivo differentiated mature MC may represent targets of hantaviruses. Human MC were isolated as described elsewhere (Babina et al., 2004). Briefly, male foreskin or female breast skin tissues were treated overnight at 4 °C with dispase (0.5 mg ml⁻¹; Boehringer Mannheim). Thereafter, the epidermis was removed and dermis was mechanically minced and digested with collagenase (10 mg ml⁻¹, type 4; Worthington), hyaluronidase (5 mg ml⁻¹, type 1S; Sigma) and DNase I (10 μg ml⁻¹; Roche) in 5 mM MgSO₄ for 1 h at 37 °C. MC were purified with anti-c-kit (CD117)-coated microbeads (Miltenyi Biotec). Purified cells were cultured in basal Iscove’s medium (Biochrom) with 10% fetal calf serum, α-monothioglycerol (226 μM; Sigma), amphotericin B (2.5 μg ml⁻¹; Sigma) and stem cell factor (100 ng ml⁻¹; Peprotech). To control purity of the preparation, MC were stained with toluidine blue before and after enrichment (Fig. 1). All MC used in the presented study were examined accordingly and generally contained >99% MC (data not shown).

Integrin-β1 and integrin-β3 were reported to mediate uptake of pathogenic and non-pathogenic hantaviruses, respectively (Gavrilovskaya et al., 1998). Interestingly, high levels of both of these integrin receptor subunits were found on purified skin MC by FACS analysis performed as described previously (data not shown; Handke et al., 2009). These results were consistent with studies with primary or immortalized MC (Brown et al., 2006; Kuchler et al., 2006).

Infection studies were performed with the pathogenic Hantaan 76-118 (HTNV) and with Prospect Hill virus type-3571 (PHV), which is considered to be a non-pathogenic hantavirus. Virus stocks were produced in Vero cells as described previously (Rang et al., 2006). MC (3·10⁶) were inoculated with hantaviruses at an m.o.i. of 0.5 in a final volume of 0.5 ml for 1 h at 37 °C. Thereafter, cells were cultured in 3 ml basal Iscove’s medium at 37 °C. Six days later cells were harvested, washed in PBS and then transferred to adhesion slides for investigation of viable cells (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). After attachment to the slides for 15 min, cells were fixed and permeabilized with methanol/acetone (1:3) for 10 min at 4 °C. To test susceptibility of MC to infection, expression of intracellular hantavirus nucleocapsid protein and c-kit on the surface of the cells were examined with a hantavirus-specific polyclonal rabbit antiserum as described previously (Handke et al., 2009) and the c-kit-specific monoclonal antibody YB5.B8 (Welker et al., 2000), respectively.

As shown in Fig. 1, the experiments revealed expression of PHV and HTNV nucleocapsid proteins in cells positive for c-kit, which is a reliable MC marker. In three independent experiments with MC from three different individuals HTNV nucleocapsid was found in about 1–10% and PHV in about 1–5% of the examined MC (data not shown).

To further analyse permissiveness of MC to infection with hantavirus, production of viral plus- and minus-strand RNA was quantified in infected MC by strand-specific RT-qPCR. The method is based on virus and strand-specific RT-primers tagged with an unrelated sequence (primers are listed in Supplementary Table S1, available in JGV Online). The unrelated sequence is targeted by one of the two primers used for subsequent real-time PCR in a LightCycler 1.2 (Roche) with the Platinum SYBR Green qPCR SuperMix-UDG plus 5 μg BSA ml⁻¹ as recommended by the manufacturer (Invitrogen). The unrelated sequence-tag is essential to identify primer-directed RT-products in the pool of RT-products, which can also be generated in the absence of added RT-primers (data not shown). RNA was reverse transcribed with Superscript III RT as described in the supplied protocol (Invitrogen). To increase the specificity of the primer-directed reverse transcription the reaction was performed at 60 °C. By these means in vitro transcribed PHV plus-strand RNA was detected with the plus-strand-specific RT-primer with an efficiency that was at least 600-fold higher compared with the RT-PCR performed with the corresponding minus-strand RT-primer. Vice versa viral RNA derived from PHV virus secreted into the medium of infected Vero cells was amplified with about 12-fold higher efficiency in samples with the minus-strand compared with the plus-strand.
specific primers. This result suggests that medium of infected cells might contain not only virion-associated RNA, but also plus-strand RNA released from infected cells. This assumption was supported by the finding that the ratio to amplify RNA with a minus-strand versus a plus-strand-specific RT-primer increased from twofold to 45-fold using RNA derived from HTNV stocks and from HTNV purified through a 30% sucrose cushion, respectively. Taken together, these results demonstrate that the applied technique allows sequence specific amplification of viral plus- and minus-strand RNA.

To analyse production of these products in infected MC, total RNA was isolated 1 and 2 days post-infection (p.i.). Furthermore, samples were harvested 1 h after the addition of the virus to the cells to determine the input ratio between plus- and minus-strand RNA. In the latter samples the relative amount of plus-strand RNA was about 16-fold and twofold lower compared with the minus-strand of HTNV and PHV, respectively (Fig. 2a, 1 h p.i.). The reason for this difference is unclear and further studies are required to determine the impact and significance of this finding. Within the following 2 days the ratio inverted and
more than nine- and 30-fold higher levels of plus-strand compared with minus-strand HTNV RNA was found at 1 and 2 days p.i., respectively. Also in PHV-infected MC the ratio between plus-strand and minus-strand RNA inverted within the first day post-infection and the amounts of both types of RNA further increased within the next day. Inversion of the plus-/minus-strand ratio and increased RNA levels detected within the first 2 days of infection clearly demonstrate that MC are permissive for HTNV and to a lower extent also for PHV.

Next, production of infectious virions by MC was analysed. In situ matured MC were prepared from foreskin and breast skin as described above. After infection, cells were washed in PBS three times to remove input virus not attached to or internalized into the cells. Thereafter, cells were cultured in 3 ml basal Iscove’s medium. MC culture medium was collected at indicated time points and the amount of virions secreted was titrated on Vero cells as described previously (Handke et al., 2009). At 1 day p.i., no infectious virus was found (Fig. 2b). This result demonstrates that input virus not attached or internalized had been completely removed. In samples taken thereafter, increasing amounts of virions were produced. At 6 days p.i., maximal HTNV titres were found, that were more than 10-fold higher compared with PHV at this time point. Until 10 days p.i., also the PHV titre increased to similar levels as HTNV. These experiments revealed retarded growth kinetics of PHV in relation to HTNV. Irrespective of this difference, the results clearly indicate that both pathogenic and non-pathogenic hantaviruses can productively infect MC.

HTNV and PHV differentially induce innate responses in vitro (Alff et al., 2006; Geimonen et al., 2002; Handke et al., 2009). To analyse inflammatory and antiviral innate responses of MC to the infection with hantaviruses, expression levels of CCL5, beta interferon (IFN-β), MxA and ISG15 were determined by RT-PCR. Total RNA was isolated from MC at 1 and 2 days p.i. and reverse transcribed with Moloney murine leukemia virus RT (Invitrogen). PCR was performed as described above. Primers used for this standard RT-PCR are listed in Supplementary Table S2 (available in JGV Online). Results of the RT-PCR revealed induction of CCL5 and IFN-β at 1 day p.i. and enhanced expression of these transcripts 1 day later in PHV-infected samples (Fig. 3). CCL5 was induced also by HTNV, but the induction was not detected before 2 days p.i. Expression of the IFN inducible genes MxA and ISG15 was found at 2 days p.i. in PHV-infected but not in HTNV-infected cells within this time frame. The faint IFN-β-specific signal at 2 days p.i. suggests that HTNV can also trigger antiviral responses in MC albeit later compared with PHV. Interestingly, early induction of innate antiviral responses triggered by PHV compared with HTNV in MC inversely correlated with the level of replication as determined by quantification of the viral RNA and amount of infectious virus produced within the first phase of infection (Fig. 2).

![Graph](http://vir.sgmjournals.org/1259)

**Fig. 2.** HTNV and PHV replicate in mature human MC. MC were isolated and infected with HTNV or PHV as described in the legend to Fig. 1. (a) At indicated hours (h p.i.) and days post-infection (days p.i.) cells were harvested. From these cells total RNA was isolated and viral plus- and minus-strand RNA was quantified via RT-PCR using virus and strand-specific RT-primers tagged with an unrelated sequence. The unrelated sequence was targeted by one of the two primers used for subsequent real-time PCR. The relative amount of viral plus- and minus-strand RNA detected at 1 and 2 days p.i. is expressed in comparison to the amount of minus-strand RNA found in the RNA isolated from MC at 1 day p.i. Samples harvested 1 h p.i. indicate the input ratio between plus- and minus-strand RNA. It has to be stressed that in all virus stocks tested generally plus-strand RNA was detectable. However, the relative amounts of plus-strand RNA were always lower compared with the minus-strand. (b) At indicated time points culture medium was harvested and the number of infectious virions secreted were titrated in Vero cells. Results of two independent experiments are presented as mean ± SD. The limit of detection was 10 focus forming units per ml (f.f.u. ml−1).

A similar differential innate response to the infection with the pathogenic HTNV and the non-pathogenic PHV is induced in primary endothelial cells and established cell lines. Furthermore differential induction of antiviral responses correlated with higher replication levels of HTNV compared with PHV (Alff et al., 2006; Geimonen et al., 2002; Handke et al., 2009). Interestingly, also in situ matured MC seem to be more susceptible to infection with HTNV than with PHV. The presented data suggest that the responsible mechanisms for the differential responses...
Fig. 3. Induction of inflammatory and antiviral responses in human MC by hantaviruses. MC were isolated and infected as described in the legend to Fig. 1. For mock treatment, MC were incubated in parallel with conditioned Dulbecco's modified Eagle's medium harvested from uninfected Vero cells cultured for 7 days. One and 2 days later total RNA was isolated for RT-PCR analysis. PCR products were produced via standard RT-PCR, resolved on an agarose gel and visualized with ethidium bromide. PCR without template was included as a control (PCR ctr.).

References


