Acute hantavirus infection induces galectin-3-binding protein

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Hantaviruses are zoonotic viruses that cause life-threatening diseases when transmitted to humans. Severe hantavirus infection is manifested by impairment of renal function, pulmonary oedema and capillary leakage. Both innate and adaptive immune responses contribute to the pathogenesis, but the underlying mechanisms are not fully understood. Here, we showed that galectin-3-binding protein (Gal-3BP) was upregulated as a result of hantavirus infection both in vitro and in vivo. Gal-3BP is a secreted glycoprotein found in human serum, and increased Gal-3BP levels have been reported in chronic viral infections and in several types of cancer. Our in vitro experiments showed that, whilst Vero E6 cells (an African green monkey kidney cell line) constitutively expressed and secreted Gal-3BP, this protein was detected in primary human cells only as a result of hantavirus infection. Analysis of Gal-3BP levels in serum samples of cynomolgus macaques infected experimentally with hantavirus indicated that hantavirus infection induced Gal-3BP also in vivo. Finally, analysis of plasma samples collected from patients hospitalized because of acute hantavirus infection showed higher Gal-3BP levels during the acute than the convalescent phase. Furthermore, the Gal-3BP levels in patients with haemorrhagic fever with renal syndrome correlated with increased complement activation and with clinical variables reflecting the severity of acute hantavirus infection.

INTRODUCTION

Hantaviruses (genus Hantavirus, family Bunyaviridae) are rodent- and insectivore-borne viruses that occasionally infect humans causing either haemorrhagic fever with renal syndrome (HFRS; Eurasian hantaviruses) or hantavirus cardiopulmonary syndrome (HCPS; American hantaviruses) with respective case-fatality rates of 0.1–12 and 30–40% (Vaheri et al., 2013). Severe cases of HFRS and HCPS are manifested by impairment of renal function, pulmonary oedema and vascular leakage. The mechanisms underlying these clinical signs remain largely unknown. However, involvement of the complement system has been suggested (Sane et al., 2011). The main HFRS-causing viruses include Hantaan virus, Dobrava virus, Seoul virus and Puumala virus (PUUV). HCPS is mainly caused by Sin Nombre and Andes viruses (Jonsson et al., 2010). Tula
hantavirus (TULV) is considered a low-virulent or apathogenic hantavirus with only a few reported cases of infection (Klempt et al., 2003).

PUUV-induced HFRS is clinically characterized by high fever, headache, nausea, blurred vision, backache and abdominal pains. Proteinuria, haematuria and oliguria, followed by polyuria, indicate renal dysfunction, which is typical in HFRS (Vaheri et al., 2013). Common laboratory findings are leukocytosis, thrombocytopenia, anaemia and elevation of plasma C-reactive protein (CRP) and creatinine levels. The clinical severity of PUUV infection varies greatly, and host genes influence the clinical picture (Mäkelä et al., 2002; Mustonen et al., 1996). Complete recovery is the usual outcome. Generally, hantavirus infection leads to intense immune activation associated with the production of cytokines such as: TNF-α, IL-1, IL-5, IL-6, IL-10, IL-15, IFN-α, IFN-β, IFN-γ and RANTES/CCL5 (Borges et al., 2006; Jonsson et al., 2010; Vaheri et al., 2011). The infection also results in increased numbers of activated CD8+ T-cells and NK cells (Björkström et al., 2011; Tuuminen et al., 2007). Moreover, previous studies have shown that the extent of complement activation via the alternative pathway in PUUV infection is common and is associated with disease severity (Paakkala et al., 2000; Sane et al., 2011). In this report, we describe the upregulation of galectin-3-binding protein (Gal-3BP, previously known as Mac-2BP and also as 90K) as a result of hantavirus infection. Upregulation of Gal-3BP has been reported previously in chronic viral infections (Artini et al., 1996; Kittl et al., 2000; Longo et al., 1993; Natoli et al., 1994), but this is to our knowledge the first report of Gal-3BP induction in acute virus infection.

RESULTS

Induction of Gal-3BP by hantavirus infection in primary endothelial cells

Elevated serum levels of Gal-3BP have been reported in human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis B virus (HCV) infections (Artini et al., 1996; Natoli et al., 1994). Moreover, the transcription of Gal-3BP is, according to microarray analysis, upregulated in human umbilical vein endothelial cells (HUVECs) infected by different hantaviruses (Geimonen et al., 2002). We wanted to study whether hantavirus infection would induce the secretion of Gal-3BP in Vero E6 cells and analysed the amount of Gal-3BP released from TULV-infected and mock-infected cells. High levels of Gal-3BP were observed in supernatants from uninfected and TULV-infected cells, showing that Vero E6 cells both produce and secrete Gal-3BP independently of hantavirus infection (Fig. 1a).

Several cancer cells and other continuous cell lines over-express Gal-3BP. Therefore, we wanted to check whether infection of primary cells with pathogenic (PUUV) or apathogenic [TULV and Prospect Hill virus (PHV)] hantavirus, in contrast to the continuous Vero E6 cells, would induce Gal-3BP production. We used real-time PCR to quantify the amount of Gal-3BP mRNA produced in hantavirus-infected as compared with non-infected HUVECs. Whilst mock-infected control cells did not express Gal-3BP, the cells infected with PUUV, TULV and PHV were found to express Gal-3BP (Fig. 1b). The upregulation of Gal-3BP was observed with both apathogenic (TULV and PHV) and pathogenic (PUUV) hantaviruses. Using immunoblotting, we demonstrated that Gal-3BP upregulation occurred also at the protein level (Fig. 1c). To assess whether Gal-3BP would be secreted from infected cells, we measured the Gal-3BP levels in the supernatants by ELISA. As expected, the Gal-3BP produced as a result of hantavirus infection was secreted into the growth medium (Fig. 1d).

Finally, we wanted to study whether Gal-3BP upregulation would affect virus replication, and we used commercially available RNA interference (RNAi) to knock down Gal-3BP from these samples (Klingström et al., 2002, 2008). Serum samples (Klingström et al., 2002, 2008). Serum

Upregulation of Gal-3BP production as a result of PUUV infection in cynomolgus macaques

The observation that hantaviruses induce Gal-3BP in human endothelial cells, the primary target of hantavirus infection in vivo, prompted us to study whether upregulation of Gal-3BP would be observed in hantavirus-infected animals. In a previous study, cynomolgus macaques infected experimentally with PUUV showed symptoms mimicking human PUUV infection (Klingström et al., 2002, 2008). We set up an in-house capture ELISA for the detection of Gal-3BP from serum samples of cynomolgus macaques, and used the test to determine the Gal-3BP concentrations at different time points of the archival experimental infection samples (Klingström et al., 2002, 2008). Upregulation of Gal-3BP was seen in five of six animals challenged with PUUV (Fig. 2a, animals #4040, #4118 and #12040 in left panel; animals #53 and #59 in right panel). Gal-3BP remained at low level in passively immunized animals (Fig. 2a, animals #2190, #6114 and #11298 in left panel), even after challenge with PUUV. The upregulation of Gal-3BP was observed at around 2 weeks after inoculation (14 or 15 days). The exact kinetics of Gal-3BP induction, however, could not be determined due to the limited number of time points in the original study. We also correlated serum Gal-3BP levels with other variables measured previously from these samples (Klingström et al., 2002, 2008). Serum
Gal-3BP levels correlated positively with serum creatinine, TNF-α, IgM and IL-10 levels (Table 1). A statistically significant ($P < 0.05$) negative correlation was observed between nitric oxide and Gal-3BP levels.

**Levels of Gal-3BP in plasma of humans with acute PUUV infection**

As we observed upregulation of Gal-3BP in the serum samples of animals experimentally infected with PUUV, we decided to measure the level of Gal-3BP in the plasma samples of patients with acute HFRS. For this purpose, we used a panel of well-characterized plasma samples collected consecutively from patients hospitalized due to acute PUUV infection. The maximum plasma level of Gal-3BP during hospitalization was used for the calculation of correlations to other variables measured previously from aliquots of the same samples (Paakkala et al., 2000; Sane et al., 2011). The median maximum plasma Gal-3BP level in the acute stage of PUUV infection (7.7 μg ml$^{-1}$, range
2.1–19.3 µg ml⁻¹) was found to be significantly higher (P<0.001) than the level measured at convalescence (3.9 µg ml⁻¹, range 1.4–10.2 µg ml⁻¹) (Fig. 2b). The maximum plasma level of Gal-3BP correlated positively with the treatment time at hospital (r=0.349, P=0.007) and with variables reflecting the fluid retention during acute infection, such as change in weight during the hospital stay (r=0.321, P=0.013), the highest urinary output in the polyuric phase (r=0.307, P=0.020) and the highest systolic and diastolic blood pressure (r=0.339, P=0.007).

Table 1. Correlations of Gal-3BP levels with variables measured previously from serum samples of cynomolgus macaques infected experimentally with PUUV

<table>
<thead>
<tr>
<th>Variable</th>
<th>Spearman’s ρ</th>
<th>P value (two-tailed)</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>Creatinine</td>
<td>0.601</td>
<td>0.000</td>
<td>67</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.524</td>
<td>0.000</td>
<td>61</td>
</tr>
<tr>
<td>IgM</td>
<td>0.507</td>
<td>0.002</td>
<td>34</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.396</td>
<td>0.002</td>
<td>34</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>-0.270</td>
<td>0.027</td>
<td>67</td>
</tr>
<tr>
<td>Focus reduction neutralization test</td>
<td>0.320</td>
<td>0.065</td>
<td>34</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.305</td>
<td>0.122</td>
<td>27</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.133</td>
<td>0.307</td>
<td>61</td>
</tr>
<tr>
<td>CRP</td>
<td>0.113</td>
<td>0.361</td>
<td>67</td>
</tr>
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Spearman’s rank correlation test was used for analysis.
Increased levels of Gal-3BP are observed in tissues that are positive for viral antigen

Finally, we wanted to investigate whether Gal-3BP was upregulated in the tissues of infected animals. We stained lung, kidney, spleen and liver preparations from PUUV-infected cynomolgus macaques by immunohistochemistry, with tissues collected from uninfected animals acting as controls. These tissues have previously been stained for the presence of viral nucleoprotein (Sironen et al., 2008). Prominent, infection-associated Gal-3BP expression was seen in the bronchial and bronchiolar respiratory epithelium (Fig. 3a), in pulmonary alveolar lining cells (Fig. 3c, pneumocytes and alveolar macrophages) and in lymphocytes in the bronchus-associated lymphatic tissue (Fig. 3b). In the kidneys, glomerular mesangial cells and the tubular epithelium exhibited strong Gal-3BP staining (Fig. 3d). In spleen (Fig. 3e) and liver (Fig. 3f), no positive reaction was observed in two animals (a25, a53), whereas a positive reaction was seen in follicular lymphocytes in the spleen and liver of animal #59. As the staining was undertaken on tissues collected at 4 weeks post-infection (p.i.), a time point when clinical signs were no longer observed, it was not possible to evaluate Gal-3BP expression during the acute phase of infection. The tissues of uninfected control animals did not show any reaction (Fig. 3g–j).

**DISCUSSION**

Here, we showed that hantavirus infection induces Gal-3BP production. Initially, in vitro experiments demonstrated that Vero E6 cells (a continuous simian cell line) produced Gal-3BP irrespective of hantavirus infection; however, primary human cells (HUVECs) were found to produce Gal-3BP only after hantavirus infection. The production of Gal-3BP was accompanied by a decreased amount of viral N protein, indicating that Gal-3BP negatively affects hantavirus infection. Detection of increased Gal-3BP levels in the sera of animals (cynomolgus macaques) infected experimentally with PUUV, and in the plasmas of patients hospitalized due to acute PUUV infection, indicated that hantaviruses also induce Gal-3BP in vivo. The Gal-3BP levels were found to correlate with the levels of acute-phase proteins in the case of animals, and with complement activation and clinical severity of PUUV infection in the case of human patients. Furthermore, upregulation of Gal-3BP was also seen in the tissues of cynomolgus macaques infected with PUUV.

Although increased serum levels of Gal-3BP have been reported in several pathological conditions, ranging from chronic viral infections to cancer, no unambiguous function has been assigned to this protein (Artini et al., 1996; Darcissac et al., 2001; Grassadonia et al., 2002). Recently, Gal-3BP was reported to interact with and aggregate recombinant adeno-associated virus type 6 (Denard et al., 2012); however, the function of this interaction remains to be studied. Our unpublished results show that both

<table>
<thead>
<tr>
<th>Variable</th>
<th>Spearman’s ρ</th>
<th>P value (two-tailed)</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>Duration of hospital stay</td>
<td>0.349</td>
<td>0.007</td>
<td>59</td>
</tr>
<tr>
<td>Change in body weight during hospital stay</td>
<td>0.321</td>
<td>0.013</td>
<td>59</td>
</tr>
<tr>
<td>Systolic blood pressure, maximum</td>
<td>0.339</td>
<td>0.009</td>
<td>58</td>
</tr>
<tr>
<td>Diastolic blood pressure, maximum</td>
<td>0.401</td>
<td>0.002</td>
<td>58</td>
</tr>
<tr>
<td>Daily urinary output, maximum</td>
<td>0.307</td>
<td>0.020</td>
<td>59</td>
</tr>
<tr>
<td>Plasma TCC, maximum*</td>
<td>0.409</td>
<td>0.002</td>
<td>57</td>
</tr>
<tr>
<td>Plasma C4, maximum</td>
<td>0.342</td>
<td>0.009</td>
<td>58</td>
</tr>
<tr>
<td>Plasma C4, minimum</td>
<td>0.181</td>
<td>0.173</td>
<td>58</td>
</tr>
<tr>
<td>Plasma creatinine, maximum</td>
<td>0.246</td>
<td>0.060</td>
<td>59</td>
</tr>
<tr>
<td>Plasma CRP, maximum</td>
<td>0.166</td>
<td>0.208</td>
<td>59</td>
</tr>
<tr>
<td>Plasma IL-6, maximum</td>
<td>0.163</td>
<td>0.274</td>
<td>47</td>
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*TCC, Terminal complement complex.
apathogenic and pathogenic hantviruses (TULV and PUUV) interact with Gal-3BP. Increased Gal-3BP levels are reported in chronic viral infections of HIV, HBV, and HCV; in the case of HIV, the level of Gal-3BP has been shown to correlate with the viral load (Gröschel et al., 2000). Fairly recently, Gal-3BP expression was demonstrated to impair the infectivity of HIV (Lodermeyer et al., 2013), indicating that Gal-3BP may act as an effector of the antiviral response. However, no direct interaction between Gal-3BP and HIV has been demonstrated, and the reduction in HIV infectivity by Gal-3BP was shown to be mediated by interfering with the particle maturation (Lodermeyer et al., 2013). The finding that hantavirus-induced Gal-3BP interferes with the accumulation of N protein in endothelial cells suggests Gal-3BP also to be involved in the cellular antiviral response against hantviruses. Whilst the mechanism is still unresolved, it could be due to the ability of Gal-3BP to bind hantaviruses directly. This could take place either in the extracellular space where Gal-3BP might inhibit viral spread or in intracellular compartments where it could block virus maturation, as in the case of HIV-1 (Lodermeyer et al., 2013). In contrast, the binding of Gal-3BP to viruses could act as an alternative mode of antiviral action by triggering an innate immune response by activation of the complement system. In support of this hypothesis, we observed a correlation between complement activation and Gal-3BP production in the plasma of patients hospitalized due to acute PUUV infection.

In contrast, Gal-3BP is known to induce the expression of cytokines such as IL-1, IL-6 and TNF-α (Ullrich et al., 1994), the levels of which are also elevated during hantavirus infection (Jonsson et al., 2010). High plasma IL-6 levels have been shown to be associated with the overall severity of acute PUUV infection (Outinen et al., 2010). In the present study, Gal-3BP levels were found to have a significant correlation with serum creatinine, TNF-α, IL-10 and nitric oxide levels in blood samples of monkeys infected experimentally with PUUV. These findings indicate that Gal-3BP is indeed an acute-phase protein and might act as an indicator for pathogenesis at the cellular level. It seems likely that Gal-3BP induction would not be specific for hantavirus infection but rather would act as a general trigger for immune activation in viral (or bacterial) infections. It is intriguing to hypothesize that Gal-3BP would act as a sensor in the endoplasmic reticulum and that it might be able to recognize stress conditions of the endoplasmic reticulum (due to, for example, overexpression of viral proteins). In such conditions, the secretion of Gal-3BP could increase, thus resulting in the activation of an innate immune response. The induction and presence of Gal-3BP could also contribute directly to the pathogenesis of hantavirus infection, as it has been shown to activate T-cells and NK cells, both of which have been shown to be activated in acute hantavirus infection (Björkström et al., 2011). The fact that Gal-3BP induces the production of matrix metalloproteinase-7 (MMP-7) (Ulmer et al., 2010) could also be linked to the pathogenesis of HFRS, as the substrates of MMP-7 include collagen IV and other components of glomerular basement membranes (Thrailkill et al., 2009), whose degradation would result in increased glomerular permeability. The induction of MMP-7 production and subsequent degradation of, for example, vascular endothelial cadherin (Ichikawa et al., 2006) or release of vascular endothelial growth factor (VEGF) (Li et al., 2006) could, on the other hand, explain the disruption of the endothelial barrier and the resulting leakage of capillaries observed in HCPS and HFRS (Gorbunova et al., 2010; Mackow & Gavrilovskaya, 2009; Shrivastava-Ranjan et al., 2010). Curiously, Gal-3BP has recently been shown to induce VEGF production (Piccolo et al., 2013), and elevated levels of VEGF were reported in pulmonary oedema fluid of HCPS patients (Gavrilovskaya et al., 2012) and in the sera of patients with acute HFRS (Li et al., 2012). Interestingly, in the present study, the plasma Gal-3BP levels correlated with several variables reflecting
fluid retention during acute PUUV infection. The upregulation of VEGF in hantavirus-mediated diseases could thus be induced by Gal-3BP production.

METHODS

Ethics statement. Written informed consent was obtained from all patients, and the Ethics Committee of the Tampere University Hospital approved the study protocol. The details of the experimental PUUV infections of cynomolgus macaques have been described previously (Klingström et al., 2002, 2008).

Cells, viruses and purification of viruses. African green monkey kidney epithelial Vero E6 cells (ATCC CRL-1586) were grown and infected with an m.o.i. of 0.1–0.5 as described elsewhere (Hepojoki et al., 2010a; Huiskonen et al., 2010). HUVECs were grown in Endothelial Cell Growth Medium (MV; PromoCell) containing supplement mix (Endothelial Growth Medium Supplement Mix; PromoCell) with 40 µg gentamicin ml−1 and 250 ng Fungizone ml−1 in bottles pre-coated with gelatine at 37 °C in a humidified atmosphere containing 5 % CO2. Hantavirus infections of HUVECs were carried out in a 12-well plate at an m.o.i. of 1.

RNAi. The siRNA duplexes LGALS3BP (ID3959) Trisilencer-27 Human siRNA for in vitro knockdown of Gal-3BP were obtained from Origene. RNAi was carried out with TULV-infected HUVECs in a 12-well plate. Briefly, cells were plated to 75–90 % confluency, infected at an m.o.i. of 1 and transfected with siRNA duplexes (universal scrambled negative control and three Gal-3BP-specific siRNAs) according to the Lippofectamine RNAiMAX (Invitrogen, Life Technologies) protocol. At 3 days p.i. and approximately 72 h post-transfection, the cells were collected and analysed for Gal-3BP expression and virus production by immunoblotting. After optimization, a 5 nM concentration of siRNA duplexes was found to be optimal for HUVEC transfection.

SDS-PAGE, immunoblotting and antibodies. SDS-PAGE was performed according to standard protocols. Transfer of proteins on to nitrocellulose was carried out by wet blotting. The probing of nitrocellulose membranes was carried out as described previously (Hepojoki et al., 2010b). The primary antibodies used were: polyclonal anti-2/3N rabbit serum (Vapalahi et al., 1992), polyclonal goat anti-human Gal-3BP antibody (R&D Systems), rabbit anti-Gal-3BP serum and anti-Gal-3BP mAb clone 12D4 (Laferte et al., 2000). The secondary antibodies conjugated to anti-rabbit IR800 and anti-goat Alexa Fluor 680 (diluted 1:10000) were from Invitrogen.

Real-time PCR. A sample of mock-, PUUV-, TULV- and PHV-infected HUVECs used in immunoblotting was stored for RNA isolation in Trisure (Biolein) reagent. Isolated RNAs were transcribed to cDNA with random hexamers and real-time PCR (Stratagene MX3500P) of Gal-3BP was achieved using primer sequences described elsewhere (Park et al., 2008). Relative quantification of Gal-3BP mRNA was carried out according to the 2ΔΔCt method (Livak & Schmittgen, 2001). The mRNA levels of RNA polymerase II were used as a reference (primer sequences for RNA polymerase II: forward, 5'-GGACAGGCGGATTTGATGAT-3'; reverse, 5'-GTCCCCGAGGCTTCACTCTA-3').

Plasma assays of patients with acute PUUV infection. The patient material consisted of consecutive plasma samples from 61 hospitalized patients (44 males and 17 females, treated at the Tampere University Hospital during 2000–2004) with serologically confirmed acute PUUV infection based on PUUV IgM in a μ-capture enzyme immunoassay (Vapalahi et al., 1996). Median of four samples per patient were taken during hospital care and a single sample for each patient was collected at the convalescent phase (roughly 1 month after the onset of fever). The routine laboratory assays were determined at the Laboratory Center of the Tampere University Hospital. The complement analysis was performed by measuring SC5b-9 concentrations using an ELISA kit (Quidel), and Gal-3BP levels in plasma were determined using a Human Gal-3BP Platinum ELISA (Bender MedSystems) according to the manufacturer’s protocol.

Serum and tissue samples from PUUV-infected cynomolgus macaques. The samples used in this study were collected and analysed for cytokines and acute-phase proteins during experimental PUUV infection as reported previously (Klingström et al., 2002, 2008). The tissue samples had previously been stained positive for PUUV N protein by immunohistochemistry (Sironen et al., 2008).

ELISA for measurement of primate Gal-3BP. A purified IgG fraction of rabbit anti-Gal-3BP was coated (100 µl per well, diluted 1:100 in 0.1 M NaHCO3, pH 9.3) to MaxiSorp (NUCRO/Thermo Scientific) plates overnight at 4 °C. After washing the wells with PBS + 0.05 % Tween 20 (PBS-T), the plate was blocked for 45 min at 37 °C with 2.5 % BSA in PBS (150 µl per well). The samples (diluted 1:100 in PBS with 2 % BSA) and standards (from Human Gal-3BP Platinum ELISA; Bender MedSystems) were applied in duplicate on the plate (100 µl per well), followed by a 45 min incubation at 37 °C. After three PBS-T washes, goat anti-human Gal-3BP (R&D Systems) was added (100 µl per well, diluted 1:400 in PBS with 2 % BSA), followed by a 45 min incubation at 37 °C and three PBS-T washes. HRP-conjugated rabbit anti-goat (DakoCytomation) was applied to the wells (100 µl per well, diluted 1:1000 in PBS with 2 % BSA) and the plate was incubated for 45 min at 37 °C. After three PBS-T and a single PBS wash, detection was carried out using 3,3',5,5'-tetramethylbenzidine liquid substrate solution according to the manufacturer’s recommendations (Sigma-Aldrich). The results were read by Multiskan (Thermo Scientific) at 450 nm. The concentration of Gal-3BP in the supernatants of hantavirus-infected HUVECs was determined using the above protocol.

Statistical analyses. Statistical analyses of the levels of factors in serum and plasma samples were performed using spss software version 18. The maximum values of Gal-3BP and SC5b-9 during the acute stage were used in the analyses. Correlations between the variables were analysed with Spearman’s rank correlation test. All P values relate to two-tailed tests and statistical significance was considered at the 5 % level.

Immunohistochemistry. Samples of lung, kidney, spleen and liver were dissected, fixed in 3 % paraformaldehyde for 48 h, dehydrated and paraffin embedded. Sections of 4 µm were cut on slides prior to staining, which was performed on a fully automated Ventana Discovery Slide Stainer (Ventana Medical Systems). The Gal-3BP-specific mAb 12D4 was used to visualize Gal-3BP in tissue samples of mock- and PUUV-infected monkeys. A Ventana IHC DAB MAP kit was used for detection, and sections were counterstained with haematoxylin and post-counterstained with bluing reagent. Finally, the slides were rinsed and dehydrated before mounting with Eukitt medium.

ACKNOWLEDGEMENTS

Ms Irina Suomalainen is thanked for skilful assistance in immunohistochemical staining experiments carried out using Ventana. Professor Suzanne Laferte and Professor William G. Chaney are acknowledged for providing anti-Gal-3BP antiserum. This work was supported by the following grants: European Commission Project
(grants QLK2-CT-2002-01358 and GOCE-CT-2003-010284 EDEN), the Academy of Finland (grant no. 259376); the Sigrid Juselius Foundation (grant number 39-3554-30); Helsinki University Hospital Research Funds; the Competitive State Research Funding of the Expert Responsibility Area of Tampere University Hospital (grant no. 9P031); the Tampere Tuberculosis Foundation; the Finnish Kidney Foundation; the Orion-Farmos Research Foundation; and the Magnus Ehrnrooth Foundation.

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