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In vivo characterization of the integrin β_3 as a receptor for Hantaan virus cellular entry

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Abbreviations: mAb, monoclonal antibody; PFU, plaque-forming unit

Abstract

Binding of viruses to cell surface molecules is an essential step in viral infection. *In vitro* studies suggested that the $\alpha_v\beta_3$ integrin receptor is the epithelial cell receptor for Hantaan virus (HTNV). Whether β_3 is *in vivo* the only or central cellular receptor for HTNV infection is not known. To investigate the role of β_3 integrin for cellular entry of HTNV, we established an HTNV infection model in newborn murine pups. Infected pups died at an average age of 14.2 ± 1.1 days with high levels of viral antigen detected in their brain, lung, and kidney. Pre-injection of blocking monoclonal antibodies (mAb) specific for either β_3 or α_v prolonged survival significantly to a maximal average survival of 19.7 ± 1.5 days ($P < 0.01$) and 18.4 ± 0.9 days ($P < 0.01$), respectively. XT-199, a chemical blocker of the $\alpha_v\beta_3$ receptor also prolonged survival to 19.5 ± 1.3 days ($P < 0.01$). In contrast to these receptor blockades, anti-HTNV antibody was not only able to prolong survival, but 20% of infected pups achieved long-term survival. An anti-murine β_1 antibody comparatively prolonged survival (19.0 ± 1.2 days), suggesting that HTNV infection is partly mediated through integrin β_1 receptors as well as through β_3 receptors *in vivo*. Our data demonstrate that the β_3 receptor is important for HTNV infection *in vivo*, but also suggest that HTNV may utilize additional receptors beyond β_3 for cellular entry within an organism.

Keywords: Hantaan virus; integrin β_3; integrin β_1; receptor; viral entry

Introduction

Hantaviruses are a genus of genetically- and serologically-related viruses belonging to the family *Bunyaviridae*. This family includes the etiologic agents of two distinct disease syndromes in humans, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), which are transmitted from rodents to humans (Lee *et al.,* 1978; Nichol *et al.,* 1993). Old World hantanviruses, such as the Hantaan (HTN) (Asia), Seoul (worldwide), Dobrava (Europe), Puumala (Scandinavia and western Russia), and related viruses cause HFRS, which is characterized by fever, renal failure, thrombocytopenia and in severe cases, hemorrhagic manifestations. New World hantaviruses, Sin Nombre, Andes and related viruses (North and South America), cause HPS, which is characterized by severe acute respiratory dystunction and a mortality rate of approximately 50% (Nicole *et al.,* 1993; Song *et al.,* 1994; Lopez *et al.,* 1996).

Cellular receptor binding is the first step in viral infection. Many different cell surface molecules can serve as receptors for the attachment or entry of viruses. One commonly used receptor family utilized for viral entry is the integrin. Viruses that utilize these receptors include echoviruses 1, 8, 9, and 22 (Bergelson *et al.,* 1992; Bergelson *et al.,* 1993; Pulli *et al.,* 1997; Nelsen-Salz *et al.,* 1999), coxackievirus A9 (Roivainen *et al.,* 1994), foot-and mouse disease virus (FMDV) (Jackson *et al.,* 1997; Jackson *et al.,* 2000), papillomavirus (Evander *et al.,* 1997), adenovirus (Wickham *et al.,* 1993), adeno-associated virus type 2 (Summerford *et al.,* 1999), and rotavirus (Coulson *et al.,* 1997).

Recent data have demonstrated that integrins containing the β_3 chain may also be involved in the cellular entry of hantavirus (Gavrilovskaya *et al.,* 1998; 1999). However, to date, there is no strong evidence to indicate that this mechanism of cellular entry of hantavirus is of biological relevance *in vivo*. In the present studies, we establish a newborn murine model for HTN virus (HTNV) infection and show that β_3-containing integrins play a part in efficient HTNV infection in this model.

Materials and Methods

Virus

HTNV (strain 76-118) and Prospect Hill virus (PHV, strain 405), were propagated in Vero E6 cells (WT, Vero C1008; ATCC CRL 1586 USA), maintained in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (100 μg/L gentamycin) (Life Technologies, Inc.) at 37°C, 5% CO_2 incubator. Quantitative virus titers were measured by counting plaques on Vero E6 cell monolayers, as previously described (White *et al.,* 1982). For animal inoculation, dilutions of virus stock (1.5×10^7 PFU) were made in phosphate buffered salaine (PBS, pH 7.4, Life Technologies, Inc.) containing a final concentration of 0.75% bovine albumin and stored in aliquots at -80°C until used.

Murine HTN survival studies

A previous study demonstrated that newborn laboratory mice were susceptible to infection with HTNV, and the mortality by HTNV was age-dependent and with an inoculum 10 LD_{50}, was 100% in mice infected within 72 h of birth (Kim and McKee, 1985). Therefore, animal manipulations with HTNV under this study were performed using newborn mice less than 24 h after birth (Day 1), and as a control, mice 3 days after birth (Day 3). Outbred ICR pregnant mice were obtained to produce newborn mice (Sam Tako Inc., Korea). To evaluate the effects of blocking antibodies, newborn mice were injected intraperitoneally (*i.p.*) with a hamster anti-mouse integrin β_3 (CD61) chain-specific antibody, hamster anti-mouse integrin α_v (CD 51) chain-specific antibody or hamster anti-mouse integrin β_1 (CD29) chain-specific antibody (PharMingen,) or 50 μl of an mouse anti-HTNV sera prepared as previously described (Lee *et al.,* 1985) or LM609 (Chemicon), a murine anti-human $\alpha_v\beta_3$ monoclonal antibody that does not cross-react with the murine vitronectin receptor (Cheresh, 1987). Following adoptive transfer of antibodies of 5-40 μg (2.8-22.8 mg/kg body weight), the mice were challenged 16-18 h later by *i.p.* injection of 2,000 PFU of HTNV, strain 76-118. Additional newborn pups were pre-treated instead with a small-molecule, non-peptide-selective $\alpha_v\beta_3$-receptor antagonist XT-199 [3-(3-(3-(4, 5-dihydroimidazol 2-ylamino) propyloxyliso-xazol-5-yl) carbonylamino)-2-(phenylsulfonylamino) propionic acid, DuPont Pharmaceuticals Co.]. These pups received *i.p.* 5 mg/kg of XT-199 on the day before viral infection followed by 10 mg/kg of the antagonist for an additional 14 days. For all groups, survival rates were recorded for 5 weeks after challenge. Each set of HTNV challenges were performed on groups of 10 cohorted pups and were performed two or three times for each group. The results shown are the mean of each experiment. All animals were treated according to the Laboratory Institute Animal Control Guideline in compliance with National Institutes of Health-American Association of Laboratory Animal Control Guidelines. All the animal experiments were done in a BSL3 facility.

Indirect immunofluorescence assay (IFA)

For these studies, mice were sacrificed at 3- to 4-day intervals from days 4 to 35 (n = 2 per time point per group) after viral infection. Organs were excised aseptically and frozen at -70°C until examined. Blood samples were collected at the same time. For detection of HTNV antigen in tissues, specimens were embedded in a polyethylene glycol compound (Tissue-Tek II, Miles Laboratories). Sections were cut at 4 μm in a CryoCut II (Ames Cryostat microtome) at 25°C and fixed in cold acetone. Prepared slides were incubated with anti-HTNV mouse hyperimmune ascitic fluid, then stained for indirect immunofluorescence with fluorescein isothiocyanate (FITC)-labeled goat antimouse IgG (1:32 diluted, ICN/Cappel). Slides were examined with a Carl Zeiss-standard microscope with epifluorescence and FITC filter system (×400, 50W, Leitz Co.). IFA for blood samples was performed using HTNV infected Vero E6 cells as the antigen (Jung *et al.,* 2004). The IFA titers were expressed as the reciprocal of the highest dilution of the antisera that resulted in a specific immunofluorescence in the cytoplasm of the infected cells.

Statistical analysis

The Student's T test was use to assess the significance of the mean time of survival. Studies were done on 10 animals tested in two to three separate analyses.

Results and Discussion

Establishing a newborn pup model for HTNV infection

For control studies, newborn mice pups less than one day old (Day 1) were injected with 500 PFU to 7,500 PFU HTNV per recipient. Viral doses $\geq$ 2,000 PFU significantly decreased mice survival (Figure 1A), with accompanying body weight reduction by day 12-14 after infection (data not shown). In contrast, inoculations with less than 1,500 PFU had minimal effects on survival. Mice survival was -60% at Day 14 using an innoculum of 2,000 PFU. Accordingly, 2,000 PFU per pup HTNV recipient dose was chosen for the *in vivo* experiment as partial efficacy of receptor blocking might be appreciated by longer survival.

This model depends on the age of the pups at the time of HTNV infection. Inoculation with 2,000 PFU HTNV, even after only three days after birth (Day 3), did not significantly increase mortality over no HTNV inoculation controls, with an 80% survival rate (Figure 1B). However, use of a viral dose of 5,000 PFU in Day 3 mice decreased survival to 20% at 12-14 days (Figure 1B). These findings suggest that the mice de-

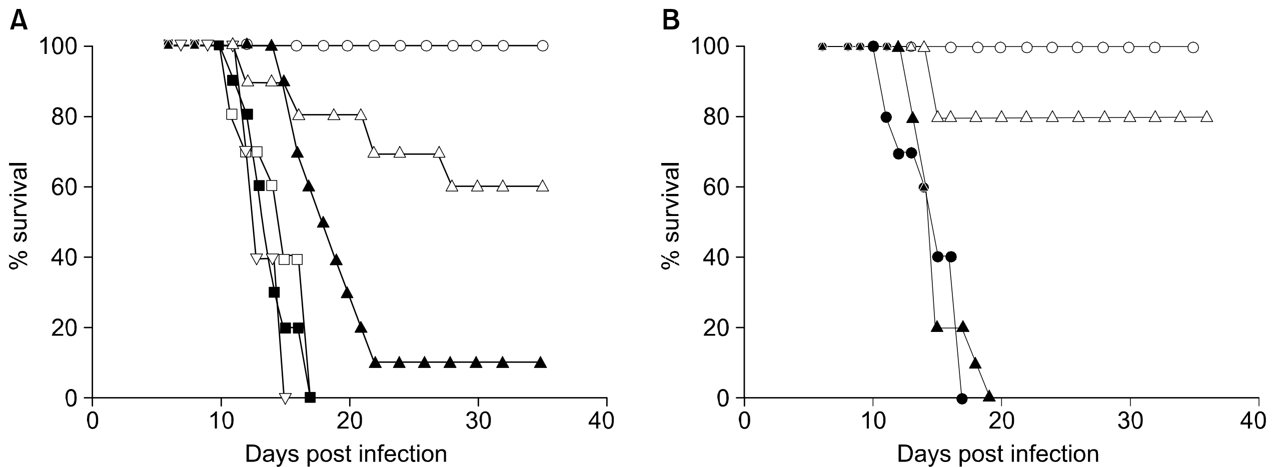


Figure 1. Percent survival rate of HTNV infected mice. Day 1 mice (10 per group) were intraperitoneally injected with (A) 1,000 (△), 1,500 (▲), 2,000 (□), 2,500 (■) and 5,000 PFU (▽) HTNV, respectively. Controls (-) were injected with PBS only without virus (○). (B) Day 3 mice (△) or Day 5 mice injected with 5,000 PFU (▲). Controls (-) (○) were injected with PBS only without virus, and controls (+) (●) were injected with 2,000 PFU of HTNV. The survival rates of HTNV infected mice were calculated daily, and the experiment was terminated on days 35. Each symbol shows mean survival value.

Table 1. Distribution of HTN viral antigen in tissue and development of antibodies in blood. ICR mice were infected intraperitoneally with 40 µl of virus (2000 PFU). Two mice from each infected group were killed on Days 7, 14, 21, 28, or 35 after infection for IFA test.

Days (p.i.)	Group	IFA titer in			
		Organ			Blood
		Brain	Lung	Kidney	
Day 7	Day 1	-	+	+	<16
	Day 3	-	-	-	<16
Day 14	Day 1	+++	+++	++	≥64
	Day 3	-	-	-	<16
Day 21	Day 3	-	-	-	2048
Day 28	Day 3	-	+	-	4096
Day 35	Day 3	-	+	-	2048

Antigen scoring: 0, no positive cells; 1+, rare positive cells; 2+, few positive cells; 3+, moderate number of positive cells; 4+, many positive cells.

velop a protective immune mechanism by three days of age and that the studies for the importance of the $\alpha_v\beta_3$ receptor on HTNV infection would need to be carried out on Day 1 mice.

The distribution of viral antigen in target organs, such as brain, lung and kidney, and the anti-HTN antibody titered in blood were determined in the Day 1 and Day 3 animals infused with 2,000 PFU (Table 1). After inoculation with HTNV on Day 1, viral antigen was detectable as early as Day 4 at low concentrations. Viral antigen levels peaked at a modest titer (≥ 64) on Day 14 in most tissues rapidly followed by the demise of the pups. These pups never developed a high titer of anti-HTNV antibody. In the Day 3 mice, none of the animals developed detectable HTNV in their tissues, but did develop high antibody titers (≥2,048), which peaked at Day 28. The modest immune response in Day 1 animals may explain the susceptibility of these animals to HTNV infection.

In vivo HTN infection blocking studies with anti-α_v and anti-β_3 antibodies

The use of antibodies to block viral binding to a specific receptor has been used successfully to identify receptors important in viral uptake (Greve *et al.,* 1989). Antibodies to β_3 integrins and the high affinity β_3 integrin ligand, vitronectin, block *in vitro* infection by pathogenic hantaviruses, but not to non-pathogenic hantaviruses. It suggested that integrin-specific interactions could contribute to hantavirus diseases (Gavrilovskaya *et al.,* 1998; 1999). Thus, CHO cell lines expressing recombinant $\alpha_{IIb}\beta_3$ or $\alpha_v\beta_3$ integrins, dramatically enhanced the infectivity of HTNV of this cell line. Pretreatment of CHO cell lines with antisera to β_3 integrins specifically reduced the number of cells infected by HTNV by > 90%. We, therefore, explored whether such antibodies would affect the course of HTNV infection in our newborn pup model and provide new insights into its *in vivo* pathobiology.

Figure 2 shows the inhibitory effect on viral toxicity of an *i.p.* infusion of the anti-mouse α_v antibody preceding infection. Mice survival rate was not appreciably different after injections at the two lowest doses of 1-2 µg (0.6-1.1 mg/kg body weight) (data not shown), but was extended in the ≥ 5 µg (2.8-22.8 mg/kg body weight) treatment group. The effect peaked at 20 µg (11.4 mg/kg body weight) anti-α_v antibody per pup compared to animals infused with PBS alone

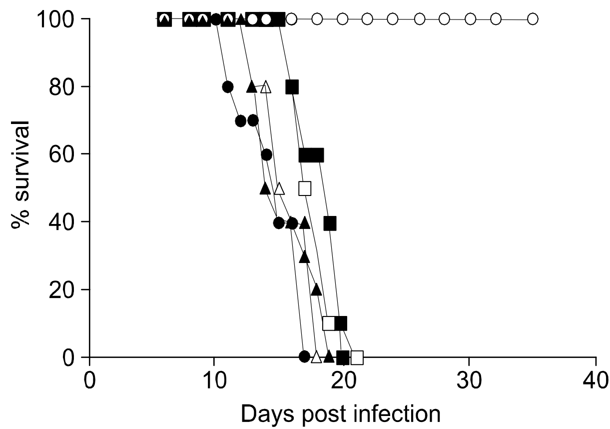


Figure 2. Percent survival rate of HTNV infected mice with mAb-α_v. Day 1 mice (10 per group) were intraperitoneally injected with 2000 PFU of HTNV, after treatment of mAb-α_v at concentrations of 5 (△), 10 (▲), 20 (□), and 40 (■) µg. Controls (-) (○) were not injected with HTN, and controls (+) (●) were injected with 2000 PFU of HTNV, but no antibody. The survival rates of HTNV infected mice were calculated daily, and the experiment was terminated on Day 35. Each symbol shows mean survival value.

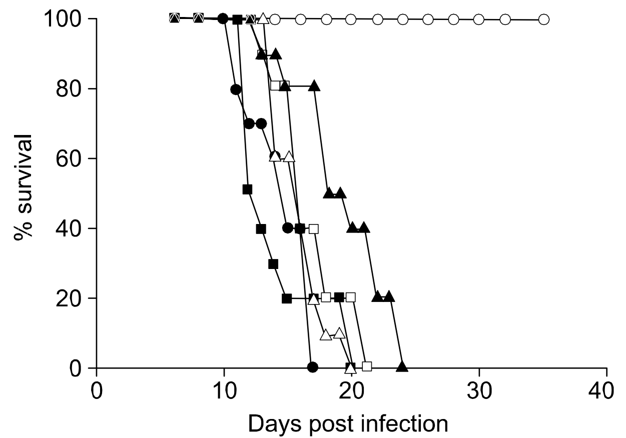
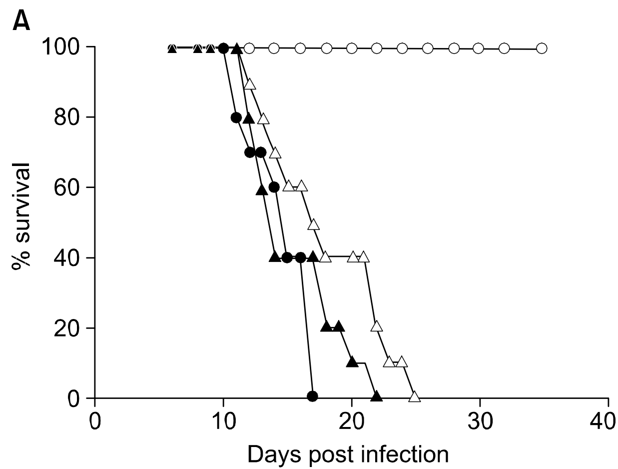
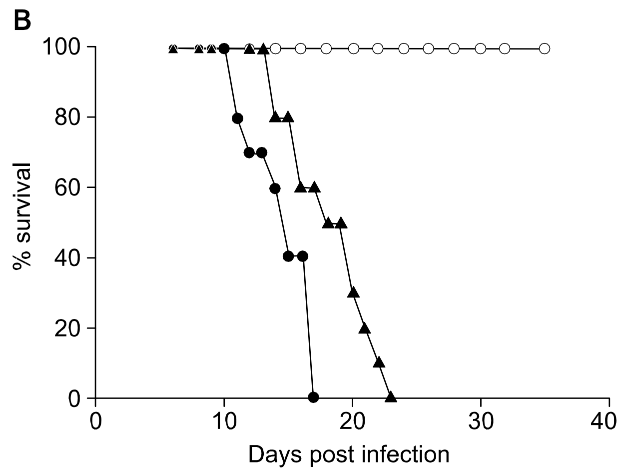


Figure 3. Percent survival rate of HTNV infected mice with anti-murine β_3 antibody. Mice (10 mice per group, Day 1) were intraperitoneally injected with 2000 PFU of HTNV, after treatment of mAb-β_3 at concentrations of 5 (△), 10 (▲), 20 (□), and 40(■) µg. Controls (-) (○) were not injected with HTNV, and controls (+) (●) were injected with 2000 PFU of HTNV, but no antibody. The survival rates of HTNV infected mice were calculated daily, and the experiment was terminated on Day 35. Each symbol shows mean survival value.





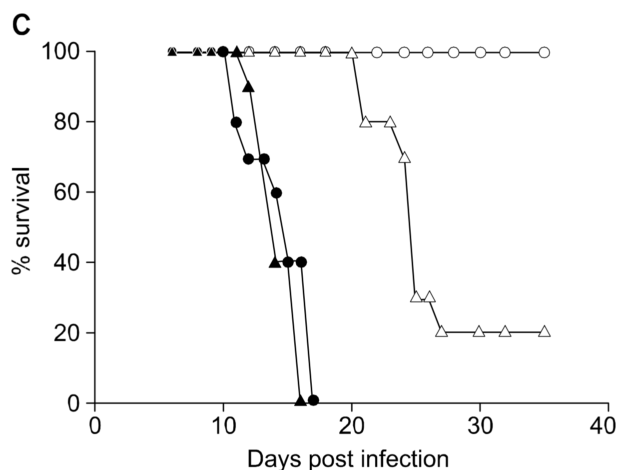


Figure 4. Percent survival rate of HTNV infected mice under various conditions. Mice (10 mice per group, Day 1) were studied as follows: (A) Controls (-) (○) were not injected with HTN, and controls (+) (●) were injected with 2000 PFU of HTNV, but no antibody. Animals were injected with HTNV after treatment of combinations of antibodies at concentrations of [each 10 µg of α_v and β_3 (△), each 20 µg of α_v and β_3 (▲)], (B) Controls (-) (○) were not injected with HTNV, and controls (+) (●) were injected with 2000 PFU of HTNV, but no antibody. Animals were injected with HTNV while being treated with XT-199(▲). (C) Controls (-) (○) were not injected with HTNV, and controls (+) (●) were injected with 2000 PFU of HTNV, but no antibody. Animals were injected with HTNV after treatment with anti-HTNV sera [anti-HTNV (△)] or LM609 (▲). The survival rates of HTNV infected mice were calculated daily, and the experiment was terminated on day 35. Each symbol shows mean survival value.

(survival: 18.4 ± 0.9 days *vs.* 14.2 ± 1.1 days, respectively, $P < 0.01$) or to animals infused with LM609, a murine anti-human $\alpha_v\beta_3$ mAb that does not react with murine $\alpha_v\beta_3$ (Cheresh, 1987) (survival: 18.4 ± 0.9 days *vs.* 14.0 ± 0.9 days, respectively, $P < 0.01$).

An anti-murine β_3 antibody was used in similar mice survival studies (Figure 3). At the lowest doses of 1 and 2 μg (0.6-1.1 mg/kg body weight) of antibody injected per pup, survival was nearly indistinguishable from that of pups injected with PBS (data not shown). However at higher antibody doses, pups survived for a longer period of time after HTNV infection, although all treated mice still succumbed by Day 23. This attenuation of the death rate was maximal at 10 μg (5.7 mg/kg body weight) of the anti-CD61 β_3 antibody per pup (survival: 19.7 ± 1.5 days *vs.* 14.2 ± 1.1 days, respectively, $P < 0.01$).

There is no murine integrin $\alpha_v\beta_3$ complex-specific antibody currently available. To further examine the significance of the integrin $\alpha_v\beta_3$ for HTN viral infectivity, combined treatment with the anti-mouse av and anti-mouse β_3 antibodies was tested (Figure 4A). We anticipated that combined therapy would have a synergistic or at least an additive protective effect. Neither was seen. At two different doses of the two antibodies, survival was prolonged for several days compared to control animals, but no difference was noted compared to treatment with the individual antibodies.

It is evident that antibody does not inhibit the virus entry in a dose dependent manner. An important finding of our studies is that in the presence of high concentration of antibodies, blocking of HTNV binding and infection by antibodies was relatively inefficient (Figure 3 and 4A). This may be because antibody have unexpected side effects by steric hinderance of other ligand-receptor interactions or only partial inhibition of the integrin signaling.

Histological samples were also examined. No difference in tissue histology for HTN viral infection was seen for the Day 1 mice for any of the blocking studies (data not shown). Additionally, there was no difference in the anti-HTNV titers that developed (data not shown). We, therefore, could not correlate the observed improvement in survival with the animals mounting a significant immune response.

Blocking the $\alpha_v\beta_3$ receptor using XT-199

The observations of a limited improvement in survival using the above anti-α_v and anti-β_3 blocking antibodies suggest that HTN viral infection is more complex than simply cell entry using the $\alpha_v\beta_3$ receptor and that one can achieve a significant, but limited, interruption in HTNV infection by blocking the $\alpha_v\beta_3$ receptor in the infectious process in newborn pups. This conclusion is supported by additional studies using a specific $\alpha_v\beta_3$-integrin receptor inhibitor XT-199 at its known blocking dose (Bishop *et al.,* 2001). In these studies, XT-199 was begun prior to HTNV infection and given for 14 days. These treated pups showed a significant improvement in survival rate compared to controls (Figure 4B) that was comparable to the improvement in survival seen with the blocking antibody studies (Figure 4A).

Ability of HTN antibody against HTN infection

Our data in Table 1 suggest that by Day 3 of life, murine pups are protected from HTN infection. These animals coincidentally develop high titers of anti-HTNV antibodies. We wondered whether it is these antibodies that are the critical difference between Day 1 and Day 3 pups in our HTNV infection model and whether the limited ability of antibodies to murine $\alpha_v\beta_3$ to protect animals may be secondary to viral infection by other mechanisms. We, therefore, *i.p.* injected anti-HTNV antibody on Day 1 of life prior to HTN infection with 2,000 PFU using an anti-HTNV titer $\geq$2,048 (Figure 4C). Anti-HTNV sera not only prolonged the overall survival rate from controls (27.0 ± 2.7 days *vs.* 14.2 ± 1.1 days, respectively, $P < 0.01$), it also resulted in the survival of 20% of the animals. Although mortality was still high, no survivors were ever seen in any tested animals using α_v or β_3 receptor blocking antibodies or drug.

These studies suggest that a major part of the reason why Day 3 mice may be protected from HTNV infection may indeed be their ability to mount a rapid and vigorous anti-viral antibody response. These data show that an antibody approach can be protective against death in this model. However, the inability of antibodies and drugs directed at the vitronectin receptor to fully protect offsprings from HTNV-related death suggest that this virus may have an alternative mechanism of cell entry during *in vivo* infection.

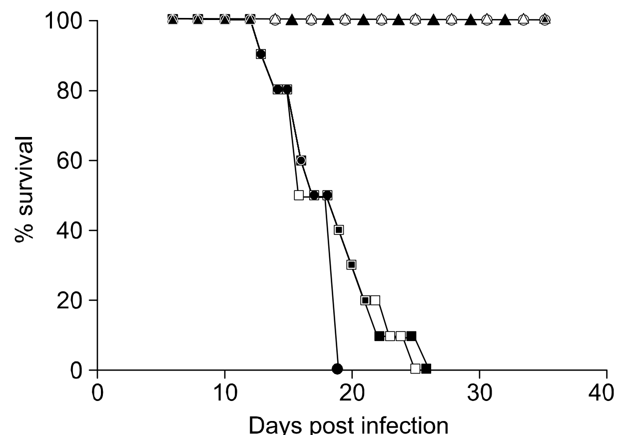


Figure 5. Percent survival rate of HTNV or PHV infected mice with anti-murine β_1 antibody. Mice (10 mice per group, Day 1) were intraperitoneally injected with 2000 PFU of HTNV or PHV, after treatment of mAb-β_1 at concentrations of 10 (□), 2 (■) μg with HTNV, and 10 (▲) μg with PHV. Controls (-) (○) were not injected with viruses, and controls (+) were injected with 2000 PFU of HTNV (●) or PHV (△). The survival rates of HTNV infected mice were calculated daily, and the experiment was terminated on Day 35. Each symbol shows mean survival value.

Such similar cases of partial inhibition have been known. Adenovirus papillomavirus, FMDV, or coxsackievirus are the examples. Anti-integrin antibodies alone can not bring about complete inhibition of cellular entry into these organisms. These viruses, which also enter cells via $\alpha_v\beta_3$ integrins, have been reported to use additional cell surface receptors (Wickham *et al.,* 1993; Roivainen *et al.,* 1994; Huang *et al.,* 1996; Evander *et al.,* 1997; Neff *et al.,* 1998). Other viruses have been reported to utilize multiple RGD-dependent integrins to initiate infection (Triantafilou *et al.,* 2001). RGD ligand motifs recognized by $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins are absent from all hantavirus proteins. Like that of rotaviruses, their entry is not blocked by RGD peptides, indicating that their interaction with the β_3 chain is independent of the integrin binding to physiologic ligands (Gavrilovskaya *et al.,* 1999; Guerrero *et al.,* 2000). Certainly, similar HTNV infection studies using the described β_3^{null} mouse (Hodivala-Dilke *et al.,* 1999) would further support our model, but these animals are presently not available. Furthermore, because of their bleeding diathesis may be problematic for doing *i.p.* injections.

Possibility of other integrins involved in HTNV infection

We, therefore, were interested in testing other receptors, focusing on related integrins. The *in vitro* observations that the nonpathogenic PHV cellular entry is associated with β_1 integrin (Gavrilovskaya *et al.,* 1999), suggest that HTNV infection might be able to use other integrins for cell entry *in vivo*. We tested the possibility of whether β_1 integrins may be involved in HTNV infection. An anti-murine β_1 antibody was used in mice survival studies against HTNV or PHV infection (Figure 5). At doses of 10 and 20 μg (5.7-11.4 mg/kg body weight) of antibody injected per pup, survival in Day 1 mice after standard HTNV infection was maximal (18.5 ± 1.1 days and 19.0 ± 1.2 days, respectively, $P < 0.01$). Days of survival rate at 20 μg of the anti-β_1 antibody per pup was significantly prolonged compared to animals infused with PBS alone (19.0 ± 1.2 days *vs.* 15.0 ± 1.4 days, respectively, $P < 0.01$). The prolongation of survival after anti-β_1 antibodies suggest that HTNV infection is partly mediated through integrin β_1 receptors as well as through β_3 receptors *in vivo*.

In summary, these studies indicate that hantaviruses clearly utilize the $\alpha_v\beta_3$ receptor for viral entry *in vivo*. These studies also suggest that this may not be the only mechanism by which hantaviruses enter cells in a living organism. Our data would, therefore, predict that blocking strategies directed against the $\alpha_v\beta_3$ receptor in the clinical care of HTNV infections may not be as efficacious as therapy directed against the HTN virion itself. Our data also suggest that β_1 intergrins are another family of receptors through which HTNV can enter cells *in vivo*. Combined studies show that the blockade of β_1 and β_3 is not synergistic (data not shown). Collectively, these data will contribute substantially to precise pathogenetic approach toward understanding the mechanism of virulence.

Acknowledgement

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