

Theme Issue Article

Organ- and endotheliotropism of Nipah virus infections *in vivo* and *in vitro*Andrea Maisner¹; James Neufeld²; Hana Weingartl²¹Institute of Virology, Philipps University, Marburg, Germany; ²National Centre for Foreign Animal Disease, Winnipeg, Canada

Summary

Nipah virus (NiV) is a highly pathogenic paramyxovirus that was first isolated in 1999 during an outbreak in Malaysia. In contrast to other paramyxoviruses NiV infects many mammalian species. Because of its zoonotic potential, the high pathogenicity and the lack of therapeutic treatment, NiV was classified as a biosafety level 4 pathogen. In humans NiV causes a severe acute encephalitis whereas in some animal hosts respiratory symptoms are predominantly observed. Despite the differences in the clinical outcome, microvascular endothelial cell damage predominantly underlies the pathological changes in NiV infections in all sus-

ceptible host species. NiV generally induces a pronounced vasculitis which is primarily characterised by endothelial cell necrosis and inflammatory cell infiltration. For future developments of specific antiviral therapies or vaccines, a detailed understanding of the molecular basis of NiV pathogenesis is required. This article reviews the current knowledge about natural and experimental infections in different mammals, focusing on the main organ and cell tropism *in vivo*, and summarises some recent studies in cell culture on the role of ephrin-B2 and -B3 receptors in NiV infection of endothelial cells.

Keywords

Nipah virus, endothelial cells, cell tropism, organ tropism, ephrin-B2

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Introduction

Epidemiology

The highly pathogenic Nipah virus (NiV) emerged for the first time in 1998 and caused an outbreak of severe febrile encephalitis among pig farmers in Malaysia and Singapore (1). A total of 276 patients were reported, with 106 fatalities (mortality rate close to 40%). NiV was isolated from a fatal human case from the village Sungai Nipah (2). The virus was identified to be a new member of the family *Paramyxoviridae*, closely related to the Hendra virus (HeV) which was isolated in 1994 after an outbreak of severe respiratory diseases in horses and humans in Australia (3, 4). Old World fruit bats of the genus *Pteropus* have been identified as the natural host of these viruses (5). Different from all other paramyxoviruses, NiV and HeV are zoonotic paramyxoviruses infecting a remarkable range of mammalian species. Dogs, cats, hamsters and guinea pigs were found to be susceptible to NiV infection besides bats, pigs and humans (5-8). Due to the high mortality rates associated with human infections and

the lack of any therapeutic or prophylactic options, NiV and HeV have been classified as biosafety-level-4 (BSL-4) pathogens.

During the first outbreak beginning in 1998, NiV was transmitted from bats to pigs, and then to humans. Since this initial outbreak NiV has re-emerged in Bangladesh and in India (Table 1). Here infections were caused by a different NiV strain (Bangladesh strain) and were characterised by a different clinical presentation and higher case fatality rates near 70% (for review see [9]). In these more recent outbreaks NiV was directly transmitted from bats to humans, either by contact to fruits or palm sap contaminated by bat secretions, by climbing contaminated trees, or by injury through infected bats. Also human-to-human and nosocomial transmissions were implicated (10-13).

The fact that neutralising antibodies to NiV were found in many *Pteropus* species (*Pteropus hypomenalus*; *Pteropus vampyrus*, *Pteropus lylei*, *Pteropus giganteus*, *Pteropus rufus*, *Eidolon dupreanum*) and also in non-*Pteropus* species (*Hipposideros larvatus*, *Scotophilus kuhlii*), from Cambodia, Thailand, Indonesia, Bangladesh, to Madagascar, clearly demonstrates that the natural NiV reservoir is widely distributed (7, 14-17). Thus,

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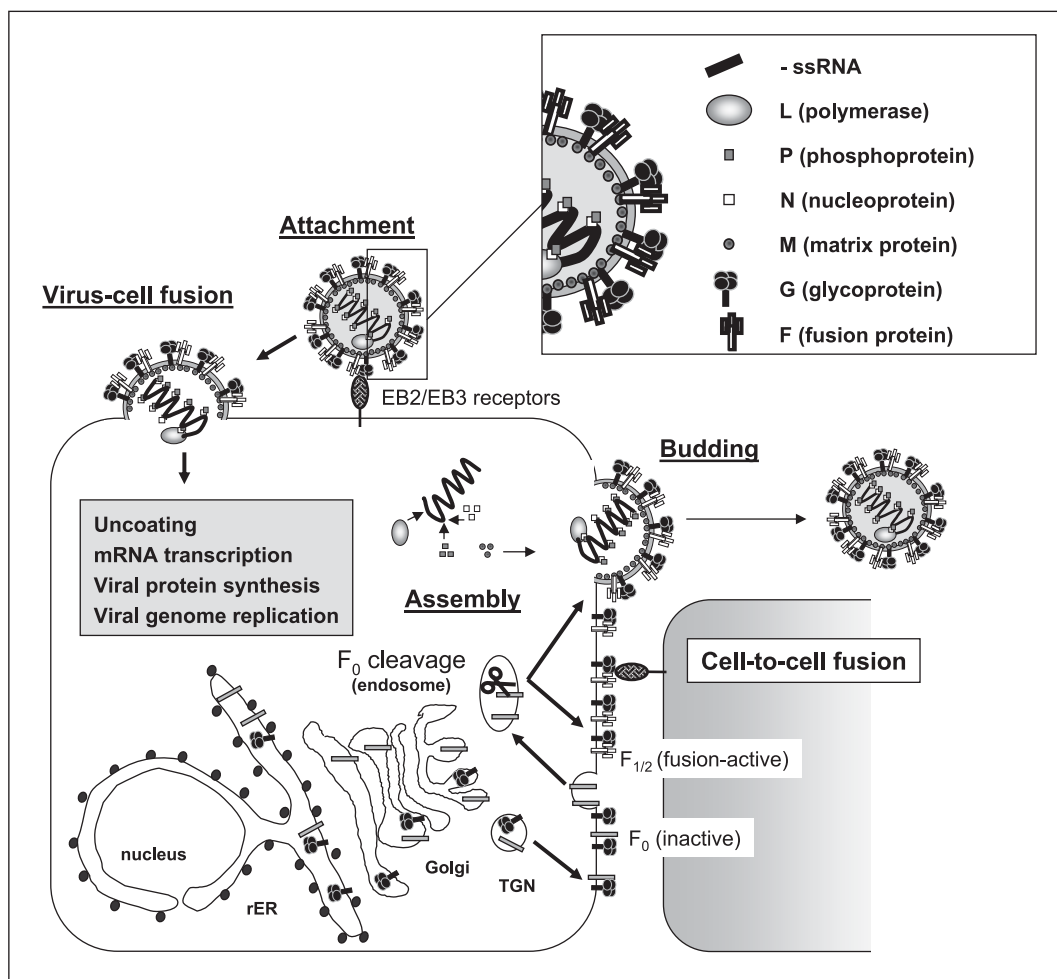


Figure 1: Schematic overview on NiV structure and replication. Abbreviations: -ssRNA, single-stranded RNA with negative sense orientation; EB2, ephrinB2; EB3, ephrinB3; F₀, uncleaved precursor fusion protein; F_{1/2}, proteolytically cleaved and fusion-active F protein.

sporadic spill-overs into the human and domestic animal population might occur in the future, also in regions outside of Malaysia, India and Bangladesh.

Virus structure and replication

NiV is an enveloped virus containing a single-stranded non-segmented RNA genome of negative polarity. Because of several unique genetic and biological characteristics NiV and the closely related Hendra virus form the new genus *Henipavirus* within the *Paramyxoviridae* family (18). As typical for paramyxoviruses, the henipavirus genome consists of six structural genes (N-P-M-F-G-L). However, several genetic features distinguish henipaviruses from other paramyxoviruses, such as unique genus-specific 3' leader and 5' trailer sequences (19), and the larger viral genome. The increased genome length of NiV (18,246 nucleotides for the Malaysian NiV strain, 18,252 nucleotides for Bangladesh NiV strain) is due to an extended open reading frame for the P gene and to longer 3' non-coding regions for all the genes except the polymerase (L) gene (4). The viral RNA genome is tightly encapsidated by the viral nucleoprotein (N), the phosphoprotein (P) and the polymerase (L), forming the ribonucleocapsid (RNP). The RNP is surrounded by the viral lipid envelope containing the three NiV envelope proteins: The

two surface glycoproteins G and F and the inner matrix protein M (Fig. 1).

The viral glycoproteins G and F are required for pH-independent virus entry into host cells. The G protein forms tetramers in the virus envelope, interacts with the NiV fusion protein F, and binds to the NiV receptors ephrin-B2 (EB2) and ephrin-B3 (EB3) on host cell surfaces (20, 21). After G-mediated receptor binding the F protein is responsible for the subsequent fusion process (Fig. 1). Besides virus-cell fusion required for virus entry the F protein also mediates fusion with adjacent cells, resulting in the formation of multinucleated syncytia if co-expressed with the G protein on the surface of infected cells or on cells expressing plasmid-encoded NiV glycoproteins (22). This glycoprotein-mediated cell-to-cell fusion influences the cytopathogenicity and allows the virus to spread directly from infected to uninfected neighbouring cells even in the absence of virus budding (23). To fulfil its important functions in fusion processes, the NiV F protein, which is synthesised in host cells as inactive precursor F₀, must be proteolytically activated by cellular proteases into the fusion-active F_{1/2} form. In that process NiV F, and also the HeV F protein, principally differs from other paramyxoviruses. Their cleavage does not depend on trypsin- or furin-like host cell proteases activating viral F proteins either during

Table 1: NiV outbreaks with human infections.

Year	Location	N° of cases	N° of fatalities	References
1997	First human NiV infections near Ipoh in Malaysia (identified retrospectively)			(1)
1998	Spread of NiV infections to Negri Sembilan. Formal recognition of the start of the NiV outbreak	265	105	(86)
1999	Spread of NiV via pig transportations to Singapore, infection of abattoir workers. May 1999, end of the first outbreak.	11	1	(43) (87)
2001	Outbreak of NiV in the Meherpur district, Bangladesh	13	9	(10)
2001	Outbreak in Siliguri, India	66	~ 50	(11)
2003	NiV outbreak in the Naogaon district, Bangladesh	12	8	(10)
2004	Outbreaks in Manikganj and Rajbari provinces, and in the Faridpur district, Bangladesh. Person-to-person transmission implicated.	42 36	14 27	(88) (89) (12)
2005	Outbreak in Tangail district, Bangladesh	12	11	(90)
2007	Outbreaks in the Nadja District, India and in Kushtia and Thakurgaon, Bangladesh. Evidence for human-to-human transmission	5 8 7	3-5 5 3	(91)
2008	Outbreaks in Manikganj and Rajbari provinces, Bangladesh	9	8	(92)

transport through the Golgi or after arrival on the cell surface. Cleavage of NiV and HeV F proteins rather requires clathrin-mediated endocytosis due to a tyrosine-based internalisation signal in its cytoplasmic tail and subsequent cleavage by endosomal cathepsin L (24-27). Only after re-transport from endosomes to the cell surface, cleaved and fusion-active F protein is available and can be incorporated into budding virions or can mediate fusion with neighbouring cells (23; Fig. 1) These exceptional requirements for proteolytic activation might be due to the fact that henipaviruses have their natural reservoir in bats whose cellular proteases might be expressed and distributed differently.

Natural and experimental NiV infection in mammals

Based on the detection of NiV antigen in the epithelial cells of several species (feline, swine), it appears that the primary replication of NiV in natural infections occurs in the epithelium (e.g. oro-nasal, upper and lower respiratory) from where the virus can spread via vascular and lymphoreticular systems (28-32). Low level viremia was also detected, namely in swine, cats and guinea pigs (29, 33-35), and virus was isolated from cerebrospinal fluid (CSF) of humans and pigs (30, 36).

Endothelial cells (END) of small blood and lymphoid vessels are the major virus target in all NiV susceptible host species. The smooth muscle cells of the tunica media are also often involved, and the infection of the vascular system is thought to lead to an extravascular spread of NiV in several organs, for example in brain, lung or spleen (28, 30, 33, 34, 36, 37; Table 2).

Beside the vascular system, the lymphoreticular system appears to be another important NiV target. Lymphoid necrosis and de-

pletion were reported for humans, swine, and cats with antigen being detected in lymph nodes, spleen, and thymus (6, 28, 30, 33, 35, 38).

In a number of species, brain is the primary target organ, with a variety of cell types being infected. Beside the END of blood vessels and surrounding smooth muscles of the tunica media, viral antigen was detected in neurons, glial cells, ependymal cells, epithelial cells of the choroid plexus. Lungs are the primary target in cats, and an important target in humans and swine. Virus presence was determined also in heart, kidney, liver, uterus and placenta, depending on the host species (6, 8, 28, 30, 32-34, 36, 38; Table 2 and 3).

NiV infections in humans

In humans symptomatic NiV infection has mainly taken the form of severe acute encephalitis, and the virus was originally isolated from cerebrospinal fluid of the first human cases (1). People infected with NiV develop antibodies against the virus (10, 39), and based on serological evidence subclinical infections were reported in Malaysia and Singapore (40).

In cases where human to human transmission was documented the clinical disease developed 6 – 11 days post exposure (41, 42). The illness started in all patients with fever, followed by headache, and varying degrees of diminishing (altered) consciousness in the majority of the cases. Myalgia, cough and difficulty in breathing were present in about half of the clinical cases, and some patients suffered from nausea, vomiting and involuntary muscle movements or convulsions (10, 11, 42, 43). In fatal cases, death, likely due to severe brain stem involvement, generally occurred within one or two weeks after the onset of the disease. The neurological signs suggested both, brain damage due to infection of small blood vessels accompanied by vasculitis with thrombosis, haemorrhage and frequent adjacent necro-

Table 2: Primary clinical signs, main organ and cell targets in different species infected with NiV.

	Human	Swine	Cat	Guinea pig	Hamster	Chicken embryo
Clinical signs	encephalitis	respiratory (CNS)	respiratory	weight loss	CNS signs	NA
Brain	END / TM neurons rare: glial cells, ependyma	END / TM neurons, glial cells, arachnoid cells, ependyma, choroid plexus epithelium	END / TM in meninges and brain, neurons		END / TM, neurons, ependyma, mononuclear cells in meninges and choroid plexus	END / TM epithelial cells of choroid plexus, neurons, glial cells, ependyma
Lung	END / TM, rare bronchiolar epithelium MGC	END / TM, alveolar/ bronchiolar epithelium, macrophages, infiltrating cells (lymphocytes)	END / TM, alveolar bronchial, bronchiolar epithelium		END/TM macrophages, neutrophils, lymphocytes	END / TM
Spleen, LNs	spleen: periarteriole sheath cells, macrophages MGC	spleen, LNs: END / TM, lymphocytes, dendritic cells, MGC	spleen, LNs: END / TM, lymphocytes	spleen: END		spleen: END / TM
Kidney	END / TM	END / TM, epithelial cells	END, epithelial cells	END (also in bladder)	END / TM	END / TM, epithelium
Heart	END / TM	END / TM		END	END / TM	END / TM

END, endothelial cells; NA, not applicable; TM, smooth muscle cells of the tunica media; CNS, central nervous system; LN, lymph node; MGC, multinucleated giant cells (Note: the origin of cells denoted as MGC is often difficult to determine). References: human: (36); swine: (28); cat: (28, 32); guinea pig: (29, 30, 34); hamster: (8); chicken embryo: (37).

sis, as well as direct infection of specific groups of neurons (2, 44). This observation was supported by histopathology and detection of viral antigen by immunohistochemistry in the brain of fatal human cases (36). Haematogenous spread of NiV appears to be the main route of central nervous system (CNS) invasion in humans (36). NiV infection can take a chronic course with a late-onset encephalitis up to a couple of years following a non-encephalitic or asymptomatic infection, or a relapsed encephalitis in patients who had previously recovered from acute encephalitis. It is suspected, that Nipah virus may persist in the central nervous system of some of the infected individuals causing the neurological relapse. In the late-onset encephalitis, the target cells are neuronal only, with no vascular involvement (36, 39, 44, 45).

Natural and experimental infection in pigs

Pigs are susceptible to NiV infection and can transmit the virus to humans (1, 2, 43). Nucleotide sequences of some of the virus isolates from porcine field cases were indeed identical with the sequences obtained from human isolates (46).

NiV infection of pigs can result in a wide range of age dependent clinical signs, from asymptomatic to porcine respiratory and encephalitis syndrome. Fever (40°C and above) was a most consistent clinical sign during the 1998/1999 outbreak. Other clinical signs involved mostly the respiratory system characterised by loud “barking” cough, difficulty breathing and mucous/yellow/bloody frothy discharge from the nose. Involvement of the nervous system was rare, with signs such as trembling, head pressing, agitation, rear leg weaknesses, uncoordinated gate. Abortions in sows were also observed, and some boars/sows suddenly died without apparent clinical signs. Morbidity rate in affected herds was reaching 100%, while mortality would remain low (1–5%) (47).

In the experimental infections in animals approximately 5–9 weeks of age, some piglets remained asymptomatic, while some did develop respiratory clinical signs, although less severe than in the field, represented by an increased respiratory rate and occasional mild coughing. Transient, short term increase in body temperature above normal (39.5°C and higher) was detected in most of the infected animals. The (oro)nasal, and subcutaneous routes of inoculations lead to severe central nervous system signs requiring euthanasia in proportion of animals which would represent a mortality rate about 15% higher than the mortality observed in the field (28, 30, 38).

Virus was detected in the upper and lower respiratory system, in the lymphoreticular and nervous system. In pigs, the virus appeared to invade the CNS directly via cranial nerves (in both, sick and apparently healthy animals) as well as by crossing the blood-brain barrier (Fig. 2A and B) (30). The animals developed good neutralising antibody titers (around 1280) by 16 days post infection (dpi), at which time the nasal and oropharyngeal shedding ceased (35).

Experimental infection in other animal species

The outcome of the NiV inoculation of guinea pigs (Hartley) is variable to such an extent, that this species is not considered to be a reliable infection model for protection studies, although guinea pigs were used as controls of NiV virulence in several studies. The disease is characterised by weight loss and transient fever 5–7 dpi, with either recovery or continuous weight loss and death (8, 34), (Weingartl et al., unpublished data). There are no reports on experimental inoculations of inbred strains of guinea pigs. Consequently it is not known if there would be more consistency in the disease outcome in these animals.

Table 3: Virus detection in organs, NiV shedding, antibody detection and clinical disease in different species infected with NiV.

	Infectious virus or viral RNA in organs	Viremia	Virus shedding	Antibody detection	Clinical disease (summarised)
Human	brain/CSF: virus isolation and RNA detection	NA	NA	antibodies detected in serum and CSF by ELISA	40 – 80% case -fatality rate acute encephalitis with respiratory involvement (2 – 36 days), relapse in some patients after 5 – 11 weeks, or late-onset encephalitis (mean interval 8 months)
Swine	virus isolation from brain/CSF ($10^{7.7}$ PFU/g), lung ($10^{3.4}$ PFU/g), spleen/LN ($10^{5.8}$ PFU/g)	RNA detected rare viremia	tonsils, nasal cavity ($10^{3.9}$ PFU/ml) oro-pharynx ($10^{4.3}$ PFU/ml)	SNT = 1280	1 – 5% mortality, in field mostly respiratory disease, under experimental conditions mostly encephalitis, some animals euthanised at 5–11 dpi
Cat	virus isolation from lung, spleen, kidney, placenta, uterine fluid Brain and heart: RNA positive	RNA detected rare viremia	virus shedding in urine virus detection in tonsils	SNT >256	in experimental inoculations most animals euthanised by 10 dpi; acute respiratory syndrome
Guinea pig	virus isolated from brain, lung, spleen, kidney, uterus, heart	positive viremia	shedding not detected	SNT = 1280	encephalitis, reduced pulmonary disease
Hamster	virus isolated from brain, lung, spleen, kidney, uterus, liver	not detected	virus shedding in urine	high serum titers detected by ELISA	encephalitis (CNS signs), most animals die between 5–8 dpi (intraperitoneal inoculation)
Bats	virus detected in kidney, uterus	not detected	virus shedding in urine Saliva: RNA positive	SNT >640	subclinical infections

CSF, cerebrospinal fluid; PFU, plaque forming units; SNT, serum neutralisation titer; dpi, days post infection; NA, not analysed. Comparison of human and animal NiV infections are well summarised in (93).

Since Wong et al. (8) reported that Swiss mice did not develop disease upon NiV inoculation, mice are not considered to be suitable animal models for NiV infections.

The golden hamster (*Mesocricetus auratus*) developed central nervous system signs upon intranasal or intraperitoneal inoculation, with the intraperitoneal route requiring significantly lower doses to reach 100% mortality in the infected hamsters. Low dose intranasal application of the virus led to a progressive deterioration (imbalance, limb paralysis, lethargy, breathing difficulties). Viremia was not detected in serum, however virus was present in urine and a number of organs. Survivors from the group inoculated intranasally developed high levels of neutralising antibodies (8).

Cats are highly susceptible to NiV infection, likely also in the field. In the experimental studies all inoculated animals developed respiratory disease, preceded by fever and changes in behaviour, and except for one, died or had to be euthanised due to severe respiratory distress 7–9 dpi (28, 33). The infection appears to be systemic with low viremia. One of the cats was pregnant at the time of necropsy, and NiV was detected in placenta, fetal tissues and uterine fluid (32).

Six days old chicken embryonated eggs represent an interesting model for studies on neuronal and vascular tropism of NiV, especially using the yolk sac inoculation route. The inoculation resulted in severe pathology of the central nervous system and mortality in 4–5 dpi in all embryos (37).

Based on serology, grey-headed fruit bats (*Pteropus poliocephalus*), one of the natural NiV reservoir species of bats, was

infected with NiV in the experimental studies, but did not develop clinical disease or pathological changes (34).

Endothelial tropism of NiV *in vivo*

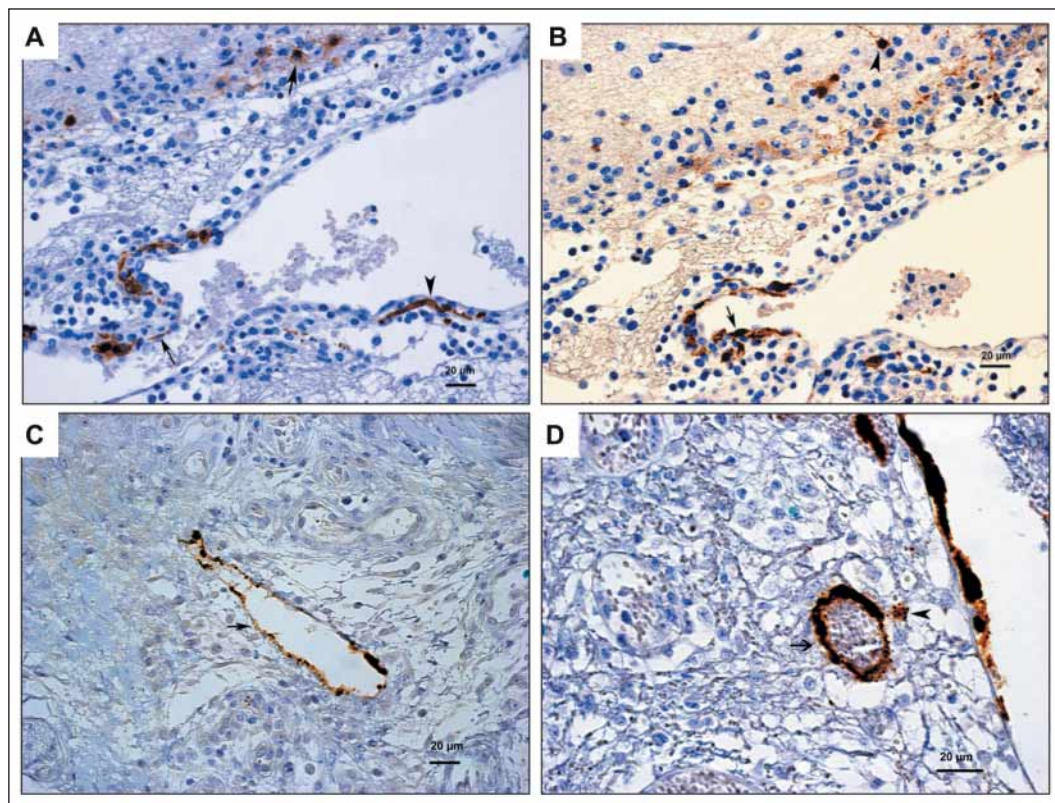
Infection of END is a hallmark of NiV infection both, in humans and in animals. It is interesting, that viral antigen was detected only in END of small blood and lymphatic vessels of the investigated host species, and there seems to be a preference for END of specific organs.

Significant involvement of small blood vessels (small arteries, arterioles, capillaries and venules) in the CNS is thought to be the basis for the development of the NiV encephalitis in humans (36, 44), hamsters (8), and cats (28, 33), and a contributing factor in the swine encephalitis (28–30). Less extensive involvement of the END of blood vessels was observed in lung, heart and kidney, and lymphatic organs, depending on the host species (Table 2).

At the microscopic level, presence of endothelial syncytia and multinucleated giant cells (even if it is difficult to identify the cell type) immunostained with anti-NiV antibodies were reported in humans, hamsters, cats, and guinea pigs infected with NiV (8, 33, 34, 36).

In swine, observation of syncytial END is rather rare, both in field and experimental specimens, although END will stain quite intensively with antibodies against NiV (Fig. 2). Interestingly epithelial syncytia are quite frequent, especially in field cases (6, 28, 29).

Figure 2: Detection of NiV antigen by immunostaining in endothelial cells of a pig experimentally infected with NiV. A) Cerebrum; NiV antigen in endothelial cells of a vessel (small arrow). Large arrow indicates an infected neuron; arrowhead points to myoepithelial cells in arachnoid in the cerebral cortex. B) Cerebrum; NiV-positive endothelial cells lining a vessel (arrow). Arrowhead indicates an infected neuron in the cortex. C) Tracheal submucosa; NiV antigen in endothelial cells of a small lymphatic vessel (arrow). D) Tracheal submucosa; NiV-positive endothelial cells of a small blood vessel (arrow) and in a tissue macrophage (arrowhead). Cell type of NiV-infected cells specifically stained in a large vessel in the right section of the figure could not be identified.



Whether the infection of END leads to necrosis or apoptosis is not known. Consequent vasculitis is characterised by various degrees of endothelial necrosis, often resulting in fibrinoid necrosis and inflammatory cell infiltration. The observed perivascular cuffing consists mostly of neutrophils and monocytes (36) but detection of lymphocytes was also reported, for example in swine (6, 30, 38).

The current understanding of involvement of END in pathogenesis of NiV *in vivo* is limited to antigen detection in fixed tissues. NiV causes vasculitis as a result of a productive infection of endothelial cells that is differently efficient in individual organs and species. In general, it is assumed that END are one of the early virus targets which contribute to the development of viremia. Frequent localisation of NiV antigen in proximity of blood (and lymph) vessels suggested that NiV-infected END increase virus load in specific organs by virus transmission to adjacent parenchymal cells. In addition, infected END may recruit immune cells, some of them also productively infected. This enhances virus invasion of the specific organ and further supports viremic spread of the virus. A model for the pathogenesis in pigs is depicted in Figure 3. Further pathogenesis models are described elsewhere (36, 48).

NiV infection of endothelial cells *in vitro*

NiV receptors on endothelial cells

EphrinB2 (EB2) is known to act as main entry receptor for NiV (49, 50), and is expressed on END, smooth muscle cells and neurons (51-55). Besides EB2, ephrinB3 (EB3) can function as

alternate receptor and is likely used in brain regions where EB2 is not expressed (56, 57). Bossart et al. (58) cloned the EB2 and EB3 genes of several NiV-susceptible species: human, pig, horse, cat, dog and bats. They found identities on the amino acid level of over 95% and demonstrated that all EB2 and EB3 molecules of all species similarly support NiV infection. This clearly shows that there is almost no variation in receptor function, regardless of species, and suggests that host receptor molecules do not play a major role in the differences in pathogenicity in different hosts.

EB2, the main NiV entry receptor is a transmembrane-anchored ligand of the receptor tyrosine kinases EphB2, EphB3 and EphB4. Interactions of Eph receptors with EB2 can trigger a wide array of cellular responses including cell adhesion, boundary formation and repulsion, and thus play a critical role in embryonic patterning, axon guidance, blood vessel remodeling and lymphangiogenesis (55, 59-61). Important for these physiological functions is the tight regulation of protein levels and an asymmetric distribution of ephrins and Eph receptors, for instance the asymmetrical arteriovenous expression of EB2 and EphB4 (51, 52, 62, 63). Eph-ephrin binding and clustering triggers a bidirectional signalling that is mediated by interactions of the cytoplasmic tails with cytosolic factors (60). In EB2, activation of the signalling cascade depends on the C-terminal 33 amino acids, and EB2 knockout or truncation of just the catalytic cytoplasmic domain resulted in a signalling-defective EB2 which had lost its ability to promote vascular remodelling (64, 65).

NiV infection in cultured endothelial cells

The capacity of END in different organs to support virus replication is thus an important determinant for the clinical outcome of NiV infection. Properties of END from different organs are known to be heterogeneous (66), and several cell- or organ-type specific host components are described to either enhance or to interfere with different steps of viral replication. However, major candidates deciding on cell tropism are clearly viral receptors. Regarding the main NiV receptor, it is known that EB2 is selectively expressed on arterial END to fulfil its function in angiogenesis and neovascularisation (52). But even if EB2 is generally expressed in arteries and arterioles, the expression levels vary greatly in different organs. Highest levels of EB2 expression were reported in lung and colon, EB2 expression in brain tissue was only in the medium range and EB2 mRNA levels detected in spleen and liver were low (67). Since this *in vivo* expression profile does not correlate with the NiV organ tropism, it remained to be determined if differences in organ-specific host factors other than receptor expression are responsible for the observed differences in END infection. To address this question, we tested the infection of different cultured model END by NiV. As *in vivo*, we found differences in the infection of END in cell culture. Inter-

estingly, only brain-derived primary or immortalised END (PBEMC [primary porcine microvascular endothelial cells]; HBEMC [human brain endothelial cells]) could be productively infected, whereas all other END types tested did not show any sign of infection (Fig. 4; upper panel). Since brain END form tight junctions and have unique transport properties, the accessibility and distribution of receptors on the cell surface may differ from END types of other organs (68). Furthermore, differential infection might be due to differences in posttranslational modifications of the receptor, such as glycosylation, which also can have profound effects on virus binding and virus-cell fusion (69). However, when we analysed EB2 and EB3 expression by RT-PCR, EB2 mRNA could only be detected in PBMEC and HBMEC, the two END types permissive to NiV infection (Fig. 4; lower panel). Interestingly, PBMEC also expressed EB3, even if EB3 was not expected to be expressed in END because it is not involved in angiogenesis (70). This result clearly demonstrated that the lack of productive NiV infection in non-brain derived cultured END is not due to differences in receptor distribution or posttranslational modifications, but is simply the result of a complete lack of receptor expression, and clearly indicates a strict correlation of EB2 expression in END and permissiveness to

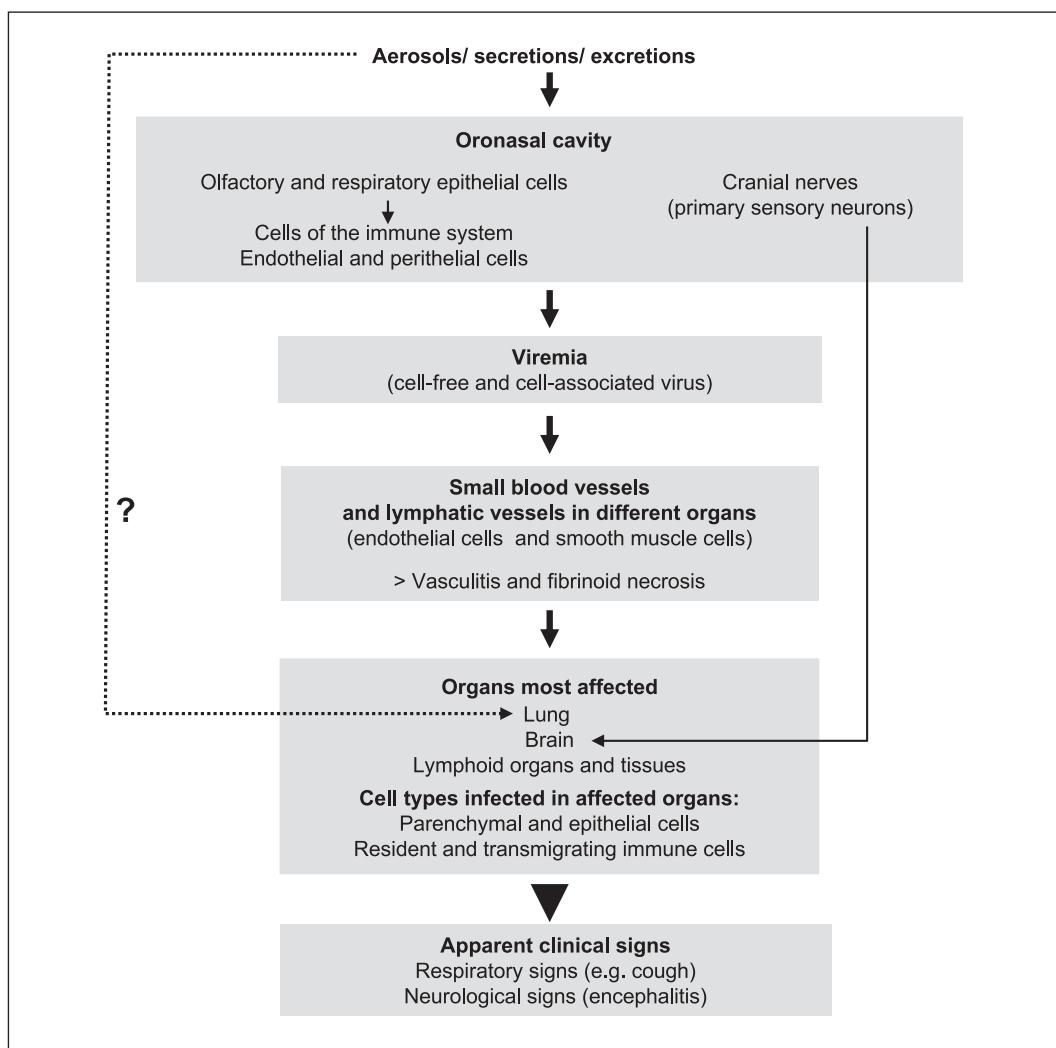


Figure 3: Model for the pathogenesis of NiV infection in pigs.

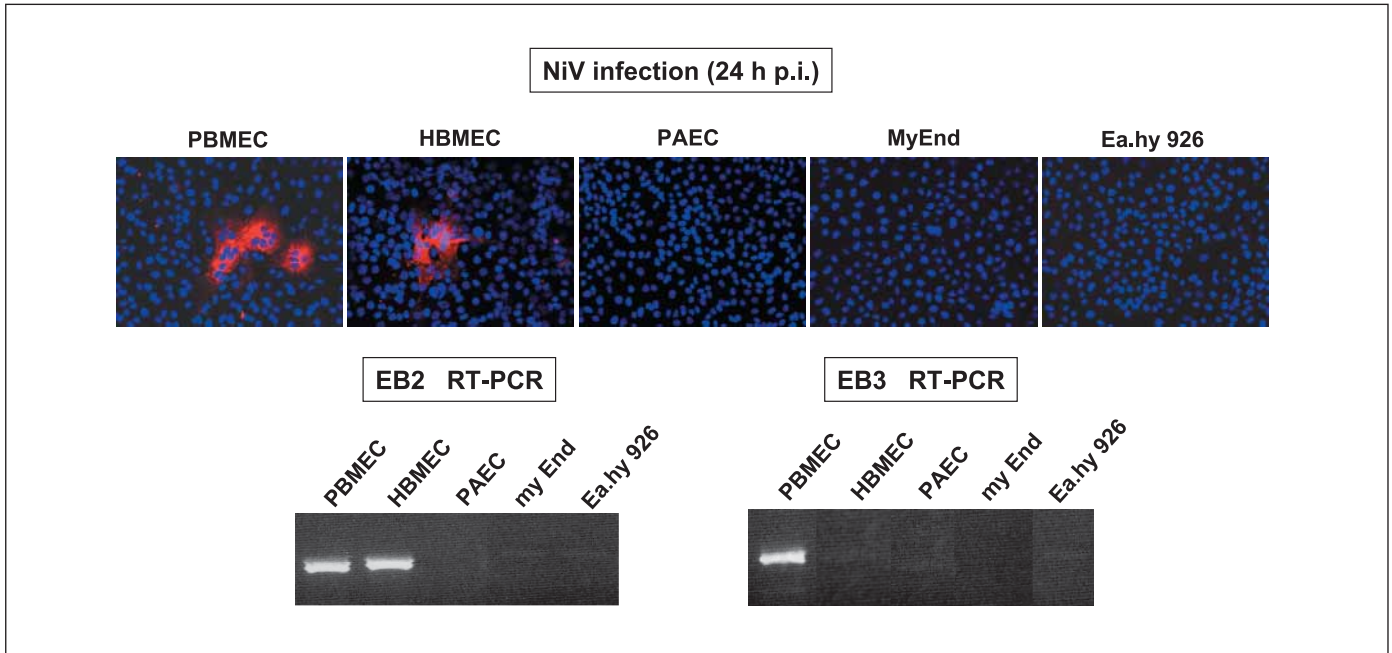


Figure 4: NiV infection and ephrin expression in different endothelial cells. Upper panel: NiV infection of different model END. PBMEC (primary porcine microvascular endothelial cells) freshly isolated from pig brain; HBMEC (human brain endothelial cells; [83]); PAEC (porcine aortic endothelial cells; [81]); MyEnd cells (mouse myocard endothelial cells) and Ea.hy 926 cells derived from human umbilical vein endothelial cells (84) were infected with NiV at a MOI of 0.2. At 24 h p.i.

cells were fixed, incubated with a NiV-specific guinea pig antiserum and virus-positive cells were visualised with rhodamine-conjugated secondary antibodies. Nuclei were counterstained with DAPI. Lower panel: EB2 and EB3 expression in model END. mRNA was extracted from 5×10^5 cells of each END type and subjected to RT-PCR with EB2 or EB3-specific primers (85).

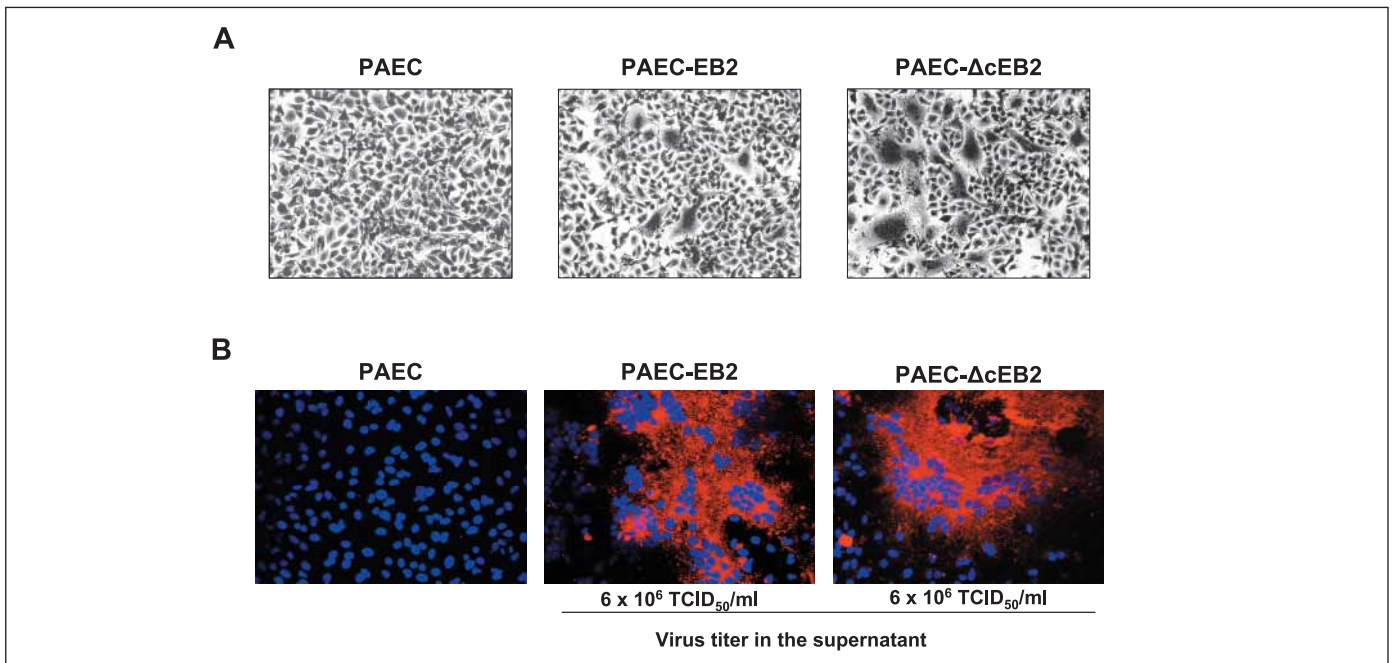


Figure 5: Receptor function of a tail-truncated EB2 ($\Delta cEB2$). A) NiV-glycoprotein mediated cell-to-cell fusion with different PAEC cells. HeLa cells were co-transfected with plasmids encoding the NiV glycoproteins F and G. Twenty-two hours after transfection, cells were mixed with normal porcine aortic endothelial cells (PAEC), or PAEC either expressing wildtype EB2 (PAEC-EB2) or tail-truncated EB2

(PAEC- $\Delta cEB2$). Twenty-four hours later, cell-to-cell fusion was visualised by Giemsa staining. B) NiV infection of different PAEC. EB2- or $\Delta cEB2$ -expressing PAEC were infected with NiV at a MOI of 0.2. At 24 h p.i. immunostaining was performed using a NiV-specific guinea pig antiserum. Cell free virus in the supernatant was determined by the TCID₅₀ method.

NiV infection in cell culture. This further suggests that cell- or organ-type specific host components such as surface-expressed C-type lectins (DC-SIGNR, LSECtin) which can promote virus attachment prior to receptor binding (71, 72), or intracellular factors influencing uncoating, viral RNA replication, viral protein synthesis or virus assembly (73–76) do not play a major role in determining which END types can be infected by NiV. Even if host factors such as interferon-induced antiviral proteins (77, 76) might influence NiV infection of END in different organs *in vivo*, these data strongly suggest that variations in the receptor expression are the important key factor for END tropism in the course of systemic NiV infections (78).

Role of tail-mediated signalling on EB2 receptor function

Since it has been shown that the Eph binding domain within EB2 and EB3 overlap with the NiV G glycoprotein binding domain, it was speculated that binding of the viral G mimics EphB3 and EphB4 binding and may lead to signalling through the ephrin ligands (56, 58). Clustering of EB2 by NiV G protein binding during virus entry might thus trigger ephrin signalling that in turn influence the entry process, for example, by proteins interacting with the catalytic domain of the EB2 cytoplasmic tail, such as proteins containing PDZ domains which can stabilise high-ordered clustering into oligomeric arrays (79). The density of this clustering or effects of EB2 signalling on actin cytoskeleton rearrangements may modulate the efficiency of virus-cell or cell-to-cell fusion (80). To evaluate this idea we analysed the capacity of a tail-truncated and therefore signalling-defective EB2 to function as NiV entry and fusion receptor. In this study we used porcine aortic endothelial cells stably expressing either wildtype

EB2 (PAEC-EB2; PAEC = porcine aortic endothelial cells) or a tail-truncated Δ cEB2 that lacks the C-terminal 67 amino acids on NiV replication (PAEC- Δ cEB2) (81). First, we compared NiV glycoprotein-mediated cell-to-cell fusion by mixing EB2-negative HeLa cells expressing plasmid-encoded F and G proteins with EB2-negative PAEC, or PAEC expressing wildtype EB2 or Δ cEB2. Since NiV G and F proteins co-expressed on the cell surface mediate cell-to-cell fusion with contacting receptor-positive cells, this assay allows testing of cell lines for functional receptor expression independent of NiV replication. As expected, receptor-negative PAEC did not fuse with F/G-expressing HeLa cells. However, syncytia formation was clearly observed in both EB2-expressing samples demonstrating that tail-truncated EB2 fully supported NiV-glycoprotein mediated cell-to-cell fusion (Fig. 5A). Similarly, we did not see a major difference when we infected PAEC-EB2 and PAEC- Δ cEB2 with live NiV and monitored the infection by quantifying the amount of virus-positive cells and virus titration (Fig. 5B). The finding that there are no substantial differences in the amount of initially infected cells, NiV-mediated cell-to-cell fusion and virus titers clearly shows that not only full-length but also tail-truncated Δ cEB2 can fully function as NiV entry receptor in endothelial cells (82). It can thus be concluded that putative signals sent through EB2 upon interaction with NiV G are not essentially required for entry or fusion processes in endothelial cells.

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