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Review

Hantavirus infections and their prevention

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ABSTRACT – Hantaviruses are rodent-borne bunyaviruses which cause haemorrhagic fever with renal syndrome and *Hantavirus* pulmonary syndrome in humans. This review covers the host interactions of the viruses, including the rodent reservoirs, the clinical outcome of human infections as well as the pathogenesis and laboratory diagnosis of infections. The current stage in prophylaxis and therapy of hantaviral diseases is described and different approaches in vaccine development are discussed.

Bunyaviridae / *Hantavirus* / rodent-borne viruses / vaccine development / virus diagnostics

1. Introduction

Hantaviruses cause two human zoonoses, haemorrhagic fever with renal syndrome (HFRS) and *Hantavirus* pulmonary syndrome (HPS). They are transmitted from rodents as their natural virus reservoir. Hantaviruses belong to the group of 'emerging viruses' [1]. There are several potential reasons for their emergence; e.g., changes in rodent population density or the invasion of humans in the environment of rodents. Certainly, the recent progress in the characterisation of the responsible agents and in the diagnosis of human infections are also decisive for the increasing number of recognised *Hantavirus* infections.

The first *Hantavirus* described, named Hantaan virus (HTNV), was isolated from the striped field mouse, *Apodemus agrarius*, by Ho-Wang Lee and co-workers in Korea [2] as a result of research efforts that started during the Korean War. Between 1951 and 1954 around 3 000 soldiers of the United Nations forces became ill from a disease subsequently called Korean haemorrhagic fever (KHF), characterised by symptoms such as high fever, chills, headache, generalised myalgia, abdominal and back pain and haemorrhagic manifestations. Severe clinical courses led to shock, renal failure and in up to 10% of the cases even to death.

D.C. Gajdusek was the first to postulate a relationship between the haemorrhagic fevers occurring in Korea, Russia and China and a disease, called nephropathia epidemica (NE), which had been described in Scandinavia since the 1930s [3]. NE, caused by Puumala hantavirus (PUUV), is characterised by a similar but generally milder clinical course when compared with KHF [4]. As in the case of KHF, an increased occurrence of NE has been connected with the conditions of land war; several reports describe nephritis outbreaks during World War II [5, 6] most probably caused by *Hantavirus* infections.

The term 'HFRS', as suggested by Gajdusek [7], was adopted by the World Health Organisation in 1983 [8] for the collective designation of clinical *Hantavirus* infections throughout Eurasia. One should, however, keep in mind that only about one-third of HTNV-infected patients develop bleeding or internal haemorrhages and that infection by PUUV usually presents without visible superficial haemorrhages [9]. The term *Hantavirus* (as a genus within the family Bunyaviridae) was introduced in 1985 for the group of HFRS-causing and related viruses by Connie Schmaljohn and co-workers [10].

In 1993, an outbreak of an acute pulmonary distress syndrome was reported in the south-west of the United States. Typically this disease was characterised by an acute onset of fever, headache, myalgia, hypovolemic hypotension and respiratory failure. The case fatality index was as high as 50%. Using primers on the basis of already known *Hantavirus* nucleotide sequences, a novel pathogenic *Hantavirus*, now named Sin Nombre virus (SNV), could be identified by RT-PCR within only a few weeks after notification of the outbreak [11]. This finding was unexpected since a pulmonary instead of nephrological manifestation was dominating and no hantaviruses leading to acute illness had been reported earlier in the Americas.

2. Virus–host interactions

2.1. Hantaviruses and their rodent reservoirs

Hantaviruses are enveloped, spherical particles of 80–110 nm in size. The virus genome consists of three segments of negative-stranded RNA; the large (L) segment

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Table I. Human pathogenic hantaviruses and their rodent reservoirs.

Host (family Muridae)			Virus (family Bunyaviridae, genus *Hantavirus*)	Disease
Subfamily	Genus	Species	Species	
Arvicolinae	*Clethrionomys*	*glareolus*	Puumala virus (PUUV)	HFRS
Murinae	*Apodemus*	*agrarius*	Hantaan virus (HTNV)	HFRS
		agrarius	Dobrava virus (DOBV-Aa)	HFRS
		flavicollis	Dobrava virus (DOBV-Af)	HFRS
	Rattus	*rattus*	Seoul virus (SEOV)	HFRS
		norvegicus	SEOV	HFRS
Sigmodontinae	*Peromyscus*	*maniculatus*	Sin Nombre virus (SNV)	HPS
		leucopus	New York virus (NYV)	HPS
	Oryzomys	*palustris*	Bayou virus (BAYV)	HPS
	Sigmodon	*hispidus*	Black Creek Canal virus (BCCV)	HPS
	Oligoryzomys	*longicaudatus*	Andes virus (ANDV)	HPS
	Calomys	*laucha*	Laguna Negra virus (LNV)	HPS

Data adapted from [37, 38, 155, 156] and Hjelle B., personal communication.

encodes the viral RNA polymerase, the medium (M) segment the envelope glycoproteins G1 and G2, and the small (S) segment the nucleocapsid protein (N). The members of the genus *Hantavirus* share morphological and genetic similarities with the other genera of the Bunyaviridae, although details of their coding strategies were found to be different [12]. Hantaviruses are the only members of the Bunyaviridae family which are transmitted to humans not by arthropods but by excretions of rodents. It has been proposed to call them roboviruses (rodent-borne viruses) in analogy to the term 'arboviruses' (arthropod-borne viruses) [13].

The genus can be divided into different *Hantavirus* species. 'Species', 'serotype', 'genotype' or simply 'hantavirus' are synonymously used terms. Pathogenicity for humans has been shown for some hantaviruses (*table I*). There is a strong association between the different *Hantavirus* species and their reservoir hosts, which indicates a co-speciation during evolution [14, 13]. Rodents harbouring hantaviruses can be classified into three subfamilies of the family Muridae; these are the Arvicolinae, Murinae, and, in the New World, Sigmodontinae subfamilies (*table I*). By phylogenetic analyses of their nucleotide sequences, the hantaviruses cluster into different genetic lineages clearly associated with their major rodent hosts [15, 16].

The major human-pathogenic Asian hantaviruses, HTNV and Seoul virus (SEOV), as well as the two human-pathogenic DOBV lineages found in Europe, are carried by Murinae rodents. These are the striped field mouse (*A. agrarius*) for HTNV, rats (*Rattus rattus* and *R. norvegicus*) for SEOV, the yellow-necked field mouse (*Apodemus flavicollis*) and the European variant of the striped field mouse (*A. agrarius*) for the two DOBV lineages, DOBV-Af and DOBV-Aa, respectively. PUUV is carried by the bank vole (*Clethrionomys glareolus*), belonging to the Arvicolinae subfamily. For other *Hantavirus* species found in Arvicolinae hosts, like Tula (TULV), Prospect Hill (PHV), Khabarovsk (KBRV) or Topografov virus (TOPV), a pathogenicity for humans has not been shown.

SNV has been identified in deer mice (*Peromyscus maniculatus*) in the south-east of the USA where the first HPS patients were found in 1993. Since then several new *Hantavirus* species have been identified in New World mice (*Sigmodontinae*) in the Americas. Human infection by *Hantavirus* species found in the USA and Canada is characterised by relatively sporadic infections, mainly by SNV. Only a few human cases have been reported which were caused by Bayou virus (host: marsh rice rat, *Oryzomys palustris*), Black Creek Canal virus (host: cotton rat, *Sigmodon hispidus*) and New York virus (host: white-footed mouse, *Peromyscus leucopus*). In South America, multiple pathogenic hantaviruses have been identified and HPS outbreaks, as well as sporadic cases, have been observed. It seems that one of the most important pathogenic hantaviruses in this continent is the Andes virus (ANDV) which is carried by the long-tailed pygmy rice rat, *Oligoryzomys longicaudatus* (*table I*). Some other genetically characterised viruses have not been isolated yet. In addition, there are hantaviruses in the Americas that have not been reported to be associated with human disease.

Hantaviruses exhibit persistent infections of their host rodents, and the evolution of the viruses should proceed in this environment [17]. In the persistently infected animal, a complex population of closely related virus variants (quasispecies) has been reported which is likely caused by point mutations [18–20]. The quasispecies occurrence may be due to evolutionary processes such as natural selection of pre-existing variants and genetic drift.

A second basis for the evolutionary development of hantaviruses is genetic changes due to RNA recombination. A first example of intramolecular recombination (within the S segment) between related lineages of TULV occurring in nature has recently been reported [21].

In addition, since hantaviruses possess segmented genomes, the possibility of genetic reassortment between different *Hantavirus* variants exists, as already known for influenza viruses. Reassortment of gene segments has been found to occur in nature between related strains of the same hantavirus species but not between different species [22, 23]. In in vitro studies Rodriguez and colleagues [24] were able to simulate genetic reassortment between two related strains of SNV but, again, they found sharply decreased reassortment probabilities between members of different species. These results suggested an

inverse correlation of genetic distances and the frequency of formation of viable virus after reassortment, which might be caused by structural and functional peculiarities of the virus genomes and their replication and, at least in nature, by the fact that hantaviruses which are primarily maintained in different rodent hosts rarely have the opportunity to genetically interact. There are indications for the occurrence of host switches in the evolution of hantaviruses; however, these seem to be rare events, whereas virus–host co-speciation remains the usual principle [25, 15].

2.2. Human diseases: HRFS and HPS

Principally, hantaviruses are transmitted to humans via aerosols of virus-contaminated rodent urine, faeces or saliva, probably also via food or hands contaminated by these excretions. In addition, there may be human cases due to rodent bites or scratches. Certain aspects of the only outbreak reported to be caused by person-to-person transmission still remain unclear [26].

The most severe forms of HFRS occur in Eastern Russia, China and Korea (caused by HTNV infection) and in the Balkan region (caused by DOBV infection). Usually, the clinical course can be subdivided into five distinct phases. After an incubation period of about 2–4 weeks, there is an abrupt onset of disease with fever, chills, general malaise, headache and other influenza-like symptoms, nausea, back and abdominal pain, gastrointestinal symptoms. This febrile phase usually lasts for 3–7 days. Towards the end of this phase, conjunctival haemorrhages and fine petechiae occur at the body surface. The hypotensive phase can last from several hours to 2 days. The characteristic drop of the blood platelet number begins. In severe cases, a clinical shock state occurs and one-third of HFRS deaths are associated with irreversible shock at this stage. In the oliguric phase (3–7 days) due to renal failure, a massive proteinuria occurs. One-half of fatalities occur during this phase. Typical findings are elevated concentrations of serum creatinine and urea. Blood pressure becomes normalised or even changes to hypertension. The onset of the diuretic phase is a positive prognostic sign for the patient. Diuresis of 3–6 L is usually observed; the daily urine production and the length of this phase (days to weeks) has been described to be correlated with the severity of the syndrome during the previous clinical phases. The convalescent phase is characterised by recovery of the clinical and biochemical markers. Usually, a restitutio ad integrum is observed.

Urban cases of HFRS in Asia but also rat-borne laboratory infections have been reported to be caused by SEOV. Most clinical cases due to SEOV infection exhibit a milder course than the above-mentioned HTNV infections. The five clinical phases of classical HFRS are sometimes difficult to define.

PUUV usually causes a rather mild form of HFRS, called NE (see above). The fatality index of NE is less than 0.2%. Analysis of about 30 clinical cases with DOBV infections in central Europe (probably caused by the DOBV-Aa lineage) shows that these patients exhibit a mild to moderate form of HFRS similar to PUUV infection ([27, 28]; our unpublished data). A single clinical case of DOBV infection with a similar clinical course was also reported from Estonia [29], a north-eastern European country were DOBV-Aa was first found [30, 31].

Early HPS symptoms resemble those of HFRS, including fever, tachypnoea and tachycardia. Thereafter, the cardiopulmonary phase progresses dramatically and makes hospitalisation and often mechanical ventilation within not more than 24 h necessary. Bilateral intestinal pulmonary oedema/infiltrates occur in combination with dyspnoea and hypoxemia. Hypotension, cardiovascular collapse and shock can be observed. Severe cardiopulmonary dysfunction predicts a poor prognosis of HPS; the case fatality is about 50%. The occurrence of aberrant lymphocytes, thrombocytopenia and pulmonary oedema are typical signs of an infection by SNV or related hantaviruses.

It should be noted that there is no absolute assignment of renal disease and HFRS on the one hand, and lung disease and HPS on the other. HPS cases with renal and/or haemorrhagic involvement especially in South America and HFRS cases with lung involvement have been observed.

There are useful comprehensive reports about the clinical courses of severe HFRS in Asia (e.g., [9]) and south-east Europe (e.g., [32]), mild HFRS/NE (e.g., [33, 34]) and HPS (e.g., [35]) as well as general surveys (for a few recent examples, see [36–38]. Certainly, several rather non-symptomatic, clinically unspecific and for other reasons undiagnosed infections exist in addition to clinically and diagnostically proven HFRS and HPS cases.

2.3. Virus- and host-dependent pathogenesis

A common factor for many of the clinical symptoms of human *Hantavirus* infections is the increased capillary permeability, which explains the haemorrhagic tendency and abdominal pain due to retroperitoneal oedema in HFRS, as well as extravasation of fluid to alveolar space and pulmonary oedema occurring in HPS [39, 36]. Hantaviruses do not cause significant cytopathic effects in vitro and viral replication should therefore not be sufficient to explain the capillary damage. The occurrence of large intracellular inclusion bodies, likely to contain viral N protein, is typical for *Hantavirus* infections [40, 41]. Cellular injury may be caused by cell-mediated immune responses; activation of $CD8^+$ T cells has been demonstrated in HPS patients, and $CD8^+$ cells together with monocytes/macrophages have been found in association with *Hantavirus*-positive lung endothelial cells [41, 42]. Human $CD8^+$ cell reactivities have been mapped to certain regions in the N proteins of both SNV and HTNV [43, 44].

Increased levels of cytokines after *Hantavirus* infection have been encountered both in vivo and in vitro. The production of tumour necrosis factor-alpha (TNF-α) can be related to symptoms observed both in HFRS and HPS, and elevated plasma levels of TNF-α as well as IL-6 and IL-10 have been reported in HFRS patients [45]. Elevated plasma/serum levels of histamine and serotonin, as well as elevated levels of nitric oxide metabolites have been described, all of which could contribute to the vascular and endothelial disturbances [46, 47].

The specific feature that enables a certain *Hantavirus* species to cause preferentially renal vs pulmonary symptoms, or subclinical/mild vs lethal manifestations, is not understood. In addition, the severity of infections by a certain *Hantavirus* species, e.g., PUUV, range from a majority of subclinical infections, to several mild cases, a few severe and sometimes even fatal cases.

In a model based on newborn mice, altered palmitoylation caused by a single amino acid exchange in the HTNV glycoprotein has been suggested to alter the virulence of the virus dramatically [48]. Similarly, different SEOV isolates can markedly differ in virulence in newborn rats [49]. Adaptation of wild-type PUUV strains to cell culture were shown to attenuate the virus for infection of its natural host. However, no changes in the genomic regions encoding the structural protein could be observed, only single mutations in the 3′ non-coding region of the S segment appeared [50]. To which extent these data from rodent models can be extrapolated to human diseases is unknown. Trials to establish a disease model simulating the human symptoms in primates had no success [51]. Although detection of viral antigen in renal tissue and mild HFRS-like manifestations have been reported in some PUUV-infected *Macaca fascicularis* animals, the significance of this model is uncertain [52].

There is evidence for a genetic susceptibility to a severe course of *Hantavirus* disease in humans, at least for PUUV infection. Of 74 HFRS patients who were HLA-typed, all cases in shock (seven out of seven) and nine out of 13 patients requiring dialysis had HLA B8 and DR3 alleles [53]. Individuals with these HLA types had a tendency to increased virus levels, as determined by viral RNA in urine and lymphocytes [54]. In contrast, patients with HLA B27 showed a preference for milder clinical courses, and moreover, only six out of 74 HFRS patients, half the figure expected, had the HLA B27 allele [55]. Recently, the biallelic polymorphisms in the TNF-α gene promoter region was studied in PUUV infected patients; the rarer TNF2 allele, associated with a high TNF-α producer phenotype, was more frequently found in hospitalised patients (42%) than in healthy controls (15%) [56].

Another important question is how the natural rodent hosts can remain persistently infected, with high titres of virus in combination with high titres of neutralising antibodies but without any notable effect on their health. Only a low frequency of the oldest individuals of natural rodent populations are sometimes antibody-positive but viral antigen/RNA-negative, probably as a sign of a past and eventually cleared *Hantavirus* infection [57, 58].

2.4. Laboratory diagnosis of infection

Since the viraemia in HFRS patients is short-termed with no more than two-thirds of acute-phase PUUV-infected patients [59, 54, 60] and about 40% of acute-phase DOBV-infected patients [61] being positive for viral RNA in the currently used RT-PCR-assays, laboratory diagnosis has to be based on serology. All common serological methods have been applied for rapid diagnosis of *Hantavirus* infections, e.g., immunofluorescence assay (IFA), haemagglutination-inhibition assay (HI), immune adherence hemagglutination assay, complement fixation test and high-density particle agglutination assay [62–69]. IFA based on viral antigen from cell culture (usually Vero E6 cells) is the classic and widely used technique for *Hantavirus* diagnostics. A variant of this methodology is the IFA IgG-avidity test, which can distinguish between virus-specific low-avidity antibodies (representing the acute phase) and virus-specific high-avidity antibodies developed during the late convalescent phase of the infection [70].

Highly sensitive and specific IgM and IgG ELISAs have also been introduced. They employ either native or recombinant N proteins as diagnostic antigens [71–75]. Application of the N protein for serodiagnosis is optimal since it induces an early and long-lasting humoral immune response [76, 72].

IgM detecting methods are important tools for diagnosing acute infections, especially in endemic areas with a high prevalence of virus-specific IgG due to previous infections. Furthermore, the majority of *Hantavirus* infected patients present IgM antibodies already at the onset of disease [72, 77, 78]. The IgG response is sometimes delayed; in one group of IgM-positive PUUV patients approximately 10% had no detectable IgG [79]; in another group, which included IgM-positive PUUV or DOBV infected patients, 12% were found IgG-negative [80]. In rare cases, also the IgM response can be delayed; in a recent study less than 2% of serum samples drawn within the first 5 days after onset of symptoms were found to be PUUV IgM-negative [81]. However, in the same serum panel, only 65% of the samples drawn within days 1–7 were IgG-positive as determined by IFA. Therefore, the optimal methodology for a rapid serological diagnosis of acute *Hantavirus* infection is based on the detection of virus-specific IgM.

The μ-capture ELISA, using viral native or recombinant N antigens, should be preferred because it is superior to IFA and solid phase IgM in terms of sensitivity, at least during the early phase of the disease [81, 82]. Follow-up sera should always be requested, if primary samples were drawn very early. It should be noted that with these highly sensitive assays, IgM can be detected in some cases as late as 2 years after the acute phase of infection [83, 72].

The humoral cross-reactivity between the different *Hantavirus* serotypes is high in humans as well as in rodents, especially within the HTNV/SEOV/DOBV-, the PUUV/PHV/TULV/TOPV-, and the SNV/ANDV-like groups. This has resulted in the conclusion that only two antigens (HTNV and PUUV) and one antigen (SNV) should be sufficient for efficient serological diagnosis of human *Hantavirus* infections in Eurasia and in the Americas, respectively. However, the recent awareness of DOBV as an important human pathogen in large parts of Europe prompted the development and evaluation of DOBV-specific serological assays ([75, 84]; our unpublished observations). The results confirmed the high degree of antibody cross-reactivity between DOBV and HTNV antigen-based ELISAs, but also proved the usefulness of homologous antigen for diagnosis/sero-epidemiology of DOBV infections; e.g., 7.1% of the DOBV-positive acute-phase patient samples and 12.5% of the DOBV convales

cent samples were found negative when examined for specific IgG against the heterologous HTNV antigen [75].

By immunoblotting, linear epitopes were shown to be mainly located on the amino-terminal part of the N protein [85, 86] and a corresponding truncated fragment was proposed as a diagnostic antigen. However, recent studies have made it obvious that the complete N protein is a more sensitive antigen compared to its terminal fragments [74, 84]. Assays utilised for sero-epidemiological screenings as well as for patient diagnosis should therefore be based on the complete N protein. During recent years, a number of recombinant *Hantavirus* antigens have been produced. Comparable studies using monoclonal antibodies (mAbs) revealed that all investigated *E. coli*-expressed PUUV N recombinants lacked one or two out of eight B-cell epitopes present in the native protein [73, 85, 87]. In contrast, HTNV, PUUV and DOBV N proteins expressed in insect cells have been found indistinguishable from the native proteins [73, 84, 88, 89]. The use of *E. coli*-expressed viral antigens required the serum samples to be adsorbed to *E. coli*-extracts to minimise non-specific reactions [90, 74]. Also, ELISAs based on direct-coating of crude preparations of native or insect cell-expressed viral antigens may suffer from non-specific reactions which can be avoided by the use of a capture-format, in which the antigen is captured by mAbs ([74]; our unpublished observations).

ELISA, or other rapid assays such as IFA or immunoblotting, which mainly detect antibodies directed against the immunodominant N protein, can not be used for serotyping since anti-N antibodies exhibit a more or less pronounced cross-reactivity between the different *Hantavirus* types. The definite infecting *Hantavirus* type can therefore only be determined either by the time- and labour-consuming neutralisation assays comparing serum titres to all relevant hantaviruses, or by RT-PCR amplification of *Hantavirus* RNA, followed by sequencing. Recent results have indicated that assays based on truncated N proteins may be suitable for differentiation of antibody responses also to closely related hantaviruses, but further evaluations are needed [91].

2.5. Risk groups and principles of infection prevention

People in contact with rodents or their excreta are at risk from *Hantavirus* infection. There are occupational risk groups of infection which exhibit higher *Hantavirus* antibody prevalences than control groups. In Germany, for instance, such risk groups include forest workers, hunters, employees of horse farms, and muskrat hunters [83]; an increased seroprevalence was also found in forest workers in The Netherlands [92] and Slovakia [93]. Other risk groups include farmers [94, 95], mammalogists [96–98], animal trappers [92] and soldiers involved in field training [99]. In case-controlled studies in the USA [100] and in Belgium [101] peridomestic cleaning of food-storage areas and animal sheds, agricultural activities such as hand ploughing, woodcutting, re-opening of non-aerated rooms and strenuous physical effort have been indicated as high-risk activities for infection and development of HPS or HFRS.

Prevention of exposition to rodents and their excreta reduces the risk of infection. Such efforts should include the elimination of rodent food sources inside the home, measures to prevent rodents entering the home as well as elimination of possible nesting sites and food sources around the home. When cleaning up potentially rodent-infested areas and rooms appropriate precautions should be taken (ventilation of the room before entering, use of rubber gloves and disinfectant, avoidance of stirring up and breathing dust). Rodents should be controlled by traps; use of gloves and masks is recommended during manipulation of traps and cadavers [37].

Measures of exposition control can reduce the individual risk of infection. However, for a more general prevention of human infections, especially in risk groups, the development of *Hantavirus* vaccines will be necessary.

3. Current stage in vaccine development and antiviral therapy

3.1. Mechanisms of protective immune response against hantaviruses

For the development of efficient vaccines the knowledge of protective viral components and the mechanisms involved in the induction of a protective immunity are important preconditions. In addition, keeping in mind the potential adverse effects of the induction of infection-enhancing antibodies [102, 103], the mapping of protective epitopes is highly important.

In the case of hantaviruses, both humoral and cellular immunity seem to be involved in protection. As expected for enveloped viruses, *Hantavirus* G1 and G2 glycoprotein derivatives are able to induce virus-neutralising antibodies in animal models which are sufficient for protection against a virus challenge (see the following sections). G1- and G2-specific mAbs have been generated which demonstrated virus-neutralising activity in vitro [104–108] as well as in vivo by passive transfer of antibodies and subsequent virus challenge in animal models ([88, 102]; our unpublished results). In addition, anti-idiotypic antibodies directed against virus-neutralising glycoprotein-specific antibodies inhibited the viral infection in cell culture [109].

There is only very limited knowledge available about the localisation of virus-neutralising epitopes, probably because of their conformational nature and dependence on glycosylation. Competition experiments with virus-neutralising mAbs and DNA vaccination of BALB/c mice with different partially overlapping segments of SNV-G1 and SNV-G2 (see *table II*) suggested the presence of several virus-neutralising epitopes both in G1 and G2 [104, 106, 110, 111]. Other epitope mapping approaches have been based on the analysis of virus immune escape mutants and truncated glycoprotein derivatives and the exploitation of a phage display strategy [112–115].

The first evidence for the role of cytotoxic T lymphocytes (CTL) in the elimination of *Hantavirus*-infected cells was obtained in BALB/c mice in vivo (adoptive transfer of spleen cells) and in vitro (chromium release assay)

Table II. Recombinant *Hantavirus* vaccine approaches.

Construct	Animal model	Challenge virus	Protection[a]	Reference
VACV- and Sindbis virus-derived live vaccines				
VACV-WR[b]-HTNV-M	hamster	HTNV	5/5	[88][d]
VACV-Con[c]-HTNV-M			4/4	
VACV-Con-HTNV-G1			(4)/5	
VACV-Con-HTNV-G2			3/5	
VACV-Con-HTNV-S			2/5	[137][g]
VACV-SEOV-M	Mongolian gerbils	SEOV	4/4	[128][e]
VACV-SEOV-S		HTNV	4/4	
		SEOV	3/4	
		HTNV	1/3	
VACV-Con-HTNV-S+M	hamster	HTNV	13/13	[138, 127][f]
		SEOV	13/13	
		PUUV	2/13	
VACV-Con-HTNV-S+M	VACV-naive hamsters	HTNV	23/24	[137][g]
	VACV-immune hamsters		23/24	
ALVAC-HTNV-M, S+M	VACV-naive hamsters	HTNV	10–85%	
NYVAC- HTNV-M, S+M	VACV-immune hamsters			
Rep5/SEOV-M	hamster	SEOV	1/5	[129][h]
Recombinant proteins				
Bac-HTNV-M	hamster	HTNV	5/5	[88][d]
Bac-HTNV-G1			3/5; (2)/5	
Bac-HTNV-G2			1/5; (4)/5	
Bac-HTNV-S			5/5	[137][g]
Bac-PUUV-N	bank vole	PUUV	8/8	[87][d]
E. coli-PUUV-N aa 1–267			3/3	
E. coli-PUUV-N aa 1–118			3/3	
E. coli-PUUV-N aa 1–79			4/5; (1)/5	
E. coli-PUUV-N aa 229–327			2/3	
Chimeric HBV core particles				
PUUV-N aa 1–45	bank vole	PUUV	8/10; (1)/10	[122, 124][d]
PUUV-N aa 75–119			1/7; (1)/7	[122, 124][d]
PUUV-N aa 1–120			7/8; (1)/8	Koletzki et al., in prep.[d]
Naked DNA vaccines and Sindbis virus-derived constructs				
pWRG/SEOV-M	hamsters	SEOV	11/11	[144][i]
		HTNV	3/4	[129][h]
pWRG/SEOV-S	hamsters	SEOV	1/14	
SNV-G1 aa 1–167	BALB/c mice	–	5/5	[110][j]
SNV-G1 aa 265–432			2/5	
SNV-G1 aa 399–565			5/5	
SNV-G1 aa 486–652			3/5	
SNV-G2 aa 1–167			4/5	
SNV-G1 aa 134–300			5/5	
pSIN2.5/SEOV-M	hamsters	SEOV	3/5	[129][h]
pSIN2.5/SEOV-S			2/8	

[a] Number of protected animals out of total; in parentheses number of partially protected animals.
[b] Mouse neurotropic strain WR.
[c] Connaught human vaccine strain.
[d] Protection measured by the absence of viral antigen in the lungs and *Hantavirus*-specific antibodies in the serum.
[e] Protection measured by the inability to re-isolate the challenge virus.
[f] Protection measured by RT-PCR of lung tissue samples (in the non-vaccinated, HTNV- and PUUV-challenged group one and three animals, respectively, were RT-PCR negative).
[g] Protection measured as the absence of viral antigen in the lungs.
[h] Protection measured as the absence of antibodies in the serum to viral proteins other than the vaccine immunogen.
[i] Protection measured by undetectable or barely detectable post-challenge anti-N antibody response and PRNT titres that remained essentially unchanged after SEOV challenge.
[j] Detection of virus-neutralising antibodies by FRNT at a dilution of 1:10.

[116–118]. The authors suggested that the CTL response was directed against the N protein [118], which was confirmed by the protection of an HTNV-infected suckling mouse against lethal infection by adoptive transfer of a T-cell enriched fraction from HTNV N protein-immunised mice [119]. Recently, $CD4^+$ cell reactivities have been mapped to four distinct regions in the N protein of PUUV [120].

In several animal models, immunisation with N protein-derived vaccines induced a protective immune response. As the N protein represents an internal protein of the virus, a cellular immune response has been favoured as the basis for providing protection. In line, N-specific mAbs or N-specific polyclonal antisera did not show any virus-neutralising activity in vitro. However, passive transfer of N protein-specific mAbs induced some protection in animal models ([119, 121]; our unpublished results) which may be caused by induction of antibody-dependent cytotoxic T cells.

The N-terminal region of the N protein of PUUV spanning aa 1–118 provided complete protection against a subsequent PUUV challenge in the bank vole model [87] (see *table II*). The major protective region of PUUV N which induces protection in 80% of the animals immunised could be mapped to aa 1–45 [122]. Interestingly, the epitope of an in vivo-protective N-specific antibody (1C12) has been mapped to this region ([123]; our unpublished observations). Further protective regions were identified between aa 75–119 [122, 124], aa 166–175 [121] and aa 229–327 [87].

Because of the presence of different *Hantavirus* serotypes in distinct geographical regions (see Sections 2.1 and 2.2) a major goal in the vaccine development is to induce a protective immunity against all relevant serotypes by the generation of multivalent vaccines. Recently, the generation of bivalent HTNV/PUUV and HTNV/SEOV vaccines has been reported [125, 126]. However, the question whether immunisation with one serotype could provide cross-protection against other serotypes is not yet completely evaluated**.** Immunisation with HTNV seems to protect, at least in part, against the SEOV serotype and vice versa [127–129]. In line, cross-reactive CTLs were identified in the mouse model and immunisation of HTNV- and SEOV-infected people with an inactivated HTNV vaccine boosted virus-neutralising antibodies against both viruses [118, 130]. An inactivated bivalent HTNV/PUUV vaccine protected hamsters against subsequent HTNV, SEOV, DOBV and PUUV, but not SNV and NYV, challenge [125]. There are conflicting data about the cross-protection between more distantly related serotypes. The adoptive transfer of T lymphocytes from PHV- and PUUV-primed BALB/c mice to nude mice induced some protection against HTNV [131] and N-protein specific CTL cell lines from HTNV-infected humans were demonstrated to be cross-reactive with distantly related serotypes [44]. In contrast, HTNV-vaccinated hamsters showed only marginal protection against PUUV [127].

3.2. Killed and recombinant live vaccines

Classically, live viral vaccines have been generated by attenuation of viruses by passaging in cell culture or animal tissues reducing the pathogenicity of the replication-competent virus vaccine. Although it has been demonstrated for hantaviruses that cultivation in Vero E6 cells resulted in a loss/decrease of infectivity for rodents due to alterations in the viral genome [50], until now there has been no attenuated *Hantavirus* vaccine available. In addition, by the development of reverse genetics for negative-stranded RNA viruses [132, 133] it should be possible in the future to generate attenuated hantaviruses with defined alterations/deletions in pathogenicity-related gene regions.

Several killed *Hantavirus* vaccines were generated by inactivation of cell- or rodent brain-passaged hantaviruses by groups in South and North Korea, China and Japan (see *table III*). A few of them are commercially produced and licensed for human use (Hantavax™ in South Korea, [125]; monovalent HTNV and SEOV vaccines in China [126]). In general, independently from the inactivation of the virus by formalin or β-propiolactone and the route of application (injection s.c. versus i.m.), after application of three doses of the inactivated vaccines a general seroconversion could be observed by IFA, ELISA and/or RPHI in nearly all vaccinees. In contrast, the induction of virus-neutralising antibodies was observed in fewer vaccinees; moreover, the titres of virus-neutralising antibodies were often found to be low, perhaps due to the destruction of the native conformation of the viral glycoproteins during the inactivation procedure or due to their low abundance in the vaccine preparations [134]. There is a necessity to improve these vaccines with respect to induction of a stronger and more long-lasting humoral immune response [135]. A major point in this respect could be the substitution of aluminium hydroxide, the only adjuvant currently allowed for human use, by more efficient adjuvants. In mice, the efficiency of an inactivated candidate SEOV vaccine (strain B1) could be improved by the adjuvant effect of two lipophilic derivatives of muramyl dipeptide [136].

The most extensive human trials with an inactivated HTNV vaccine (more than 1 200 000 people) has been undertaken in North Korea; a reduced number of HFRS cases has been observed in vaccinated populations compared with non-vaccinated controls [134]. Since the availability of the Hantavax™ in 1990, the total number of hospitalised HFRS patients in South Korea has decreased from 1 234 cases in 1991 to 687 cases in 1996. The protective efficacy of this vaccine was confirmed in a randomised, placebo controlled field study in former Yugoslavia [125]. From China, large-scale human trials have been reported recently, demonstrating a protective efficacy of 94–98% for the inactivated monovalent HTNV (Z10) and SEOV (L99) vaccines [126].

Although several of the above-described killed virus vaccines demonstrated promising protection data, there remain obvious problems concerning their production and human use. Because of the aerosol transmission, hantaviruses require high-level safety conditions for handling. The viral yield obtained from virus-infected cell cultures is quite low. At least in terms of safety and yield, recombinant subunit *Hantavirus* vaccines should be able to solve these problems.

For several hantaviruses recombinant candidate live vaccines have been generated on the basis of replication-competent vaccinia viruses or recombinant bacteria encoding single or various viral proteins. Vaccinia virus (VACV) recombinants carrying the entire M segments of HTNV and SEOV induced a complete protective immune response in the hamster and Mongolian gerbil model against HTNV and SEOV/HTNV challenge, respectively (see *table II*). In contrast, VACV recombinants carrying the

Table III. Vaccination trials with inactivated *Hantavirus* vaccines.

Inactivated vaccine (virus strain)	Vaccine generated in	Protectivity/immune responses tested in	Human trials				References
			Number of vaccinees	Delivery	Induction of		
					Sero-conversion	Virus-neutralising antibodies	
HTNV (84/105)	suckling ICR mice brain (suckling SD rat brain)	striped field mice	> 400	s.c., two doses	89–99% (IFA)	NA	[157]
			61	s.c., 1st dose 2nd dose 3rd dose	95.2% 91.7% 100% (IFA)	66.7% 75% 88.9%	[125]
			64	i.m., 1st dose 2nd dose 3rd dose	79.7%/62% 96.9%/96.9% 93.8%/100% (IFA/ELISA)	13% 75% 50%	[135]
HTNV (Z10)	Mongolian gerbil kidney cells	rabbits, Mongolian gerbils, hamsters	> 2 000	three doses	NA	90–100%	[158, 159, 134]
			> 400	three doses	35.7–53.8	NA	[160, 134]
			48	i.m., 2nd dose 3rd dose	75–100% 83–100% (RPHI, IFA, ELISA)	42–50% 92–100%	[126]
HTNV (LR1)	suckling mouse brain	rabbits, Mongolian gerbils	10	i.m., three doses	100% (at least in one assay: ELISA, RPHI, C.F., IFA)	NA	[161, 159, 134]
			200	i.m., two doses	100% (detected by ELISA, RPHI and IFA)	100% (detected by PRNT)	
HTNV (strain not reported)	suckling rat or hamster brains	NA	> 1.2 million	NA	NA	NA	[162, 134]
SEOV (R22)	suckling mouse brain	NA	30	i.m., three doses	100% (detected by ELISA and RPHI)	100% (detected by PRNT)	[163, 134]
SEOV (L99)	golden hamster kidney cells	rabbits, Mongolian gerbils, hamsters	12	i.m., two or three doses	100% (IFA, ELISA)	100%	[164]
			107	i.m., three doses	NA	97.4-100 %	[126]
SEOV (B-1)	brain of newborn ICR mice	BALB/c mice	no human trials				[165, 136]
HTNV (Z10) + SEOV (Z5)	Mongolian gerbil kidney cells	hamsters, rabbits, Mongolian gerbils	81	i.m.	NA	87.6% (HTNV) 96.3% (SEOV)	[126]
PUUV (K27) + HTNV (84/105)	suckling hamster brain/suckling mouse brain	hamsters	10	s.c., three doses	100% (HTNV and PUUV)	100% (HTNV and PUUV)	[125]

NA, data not available; i.m., intramuscularly; s.c., subcutaneously.

G1-, G2- or N-encoding sequences permitted protection only in some of the immunised animals [88, 128, 137].

A VACV recombinant vaccine carrying the S and M segment of HTNV [138] was tested in a phase I clinical study demonstrating the immunogenicity and safety of the recombinant virus. A recent phase II study revealed the induction of virus-neutralising antibodies in three out of four of VACV-naive volunteers after two immunisations, but only in approximately one quarter of VACV-immune volunteers [139]. Alternative poxvirus-derived vaccines based on NYVAC, a genetically altered VACV or ALVAC canarypoxvirus (see *table II*) were not as effective as VACV in protecting hamsters, and they have not been pursued for human use [137].

3.3. Recombinant proteins and virus-like particles

Hantavirus N protein derivatives synthesized by the use of *E. coli* and insect cell (baculovirus) expression systems [87, 88, 119, 137] have proven protective potential (see *table II*). In addition, immunisation with insect cell lysates carrying baculovirus-expressed HTNV glycoproteins G1 and G2 induced different levels of protection against a subsequent HTNV challenge in the hamster model [88].

Usually monomeric viral proteins and oligopeptides possess low immunogenicity. This disadvantage of recombinant proteins could be overcome by the generation of virus-like particles (VLPs). VLPs have been obtained for a series of different viruses by the heterologous expression and spontaneous self-assembly of viral structural proteins. Besides their non-infectivity, the main advantage of VLPs is that they resemble the structural and immunological properties of the original virus particles. In addition, the ability of several VLPs to induce a CTL response by an alternative MHC class I pathway has been demonstrated (for review see [140]).

Vaccination approaches on the basis of 'autologous' VLPs have been reported for several viruses; the first recombinant subunit vaccine licensed for human use is based on yeast-expressed HBsAg-derived VLPs [140]. However, autologous *Hantavirus* VLPs based on the heterologous co-expression of *Hantavirus* structural proteins were produced only at low level [141].

In order to exploit the advantages of VLPs in a candidate *Hantavirus* vaccine we generated chimeric VLPs based on a self-assembling carrier–*Hantavirus* fusion protein. Hepatitis B virus (HBV) core particles tolerated the insertion of the putative protective regions (aa 1–120) of PUUV (strain Vranica/Hällnäs), HTNV and DOBV in the major immunodominant region of the core ([142]; our unpublished data). Whereas the 120 aa region of PUUV N presented on core particles provided complete protection in seven out of eight animals against a PUUV challenge (our unpublished observations; see *table II*), the 45 aa region provided only partial protection in two out of five animals [124]. Interestingly, the corresponding 45 aa region of PUUV strain CG18-20 N protein completely protected 80% of bank voles [122, 124] demonstrating strain-specific peculiarities in the ability to induce a protective immune response which obviously could be overcome by using larger-sized or complete N protein sequences.

3.4. Nucleic acid vaccines

Nucleic acid vaccines, usually based on plasmid DNA, have been demonstrated as a promising vaccination strategy for various viral infections (for a review see [143]). DNA vaccination is based on the introduction of an antigen-encoding plasmid into the organism directing the de novo synthesis of the antigen in vivo. This intracellular expression of the antigen allows the induction of both humoral and cellular immune responses. DNA vaccination offers a number of advantages over live and killed vaccines. DNA vaccines are non-infectious and non-replicating. They may also provide a long-lasting immunity after a single dose application. Further advantages of DNA vaccines are their low production costs, their thermostability and the facility of multivalent vaccines.

Gene gun-mediated DNA vaccination with a plasmid bearing the SEOV M-segment cDNA was reported to induce a protective immune response in immunised hamsters [129, 144] (see *table II*). In contrast, SEOV-S-vaccinated hamsters demonstrated only some limitation of subsequent SEOV infection [129, 144]. Virus-neutralising antibodies could be induced in BALB/c mice by DNA vaccination with different SNV G1- and G2-encoding plasmids [110]. DNA vaccination with PUUV-S induced an antibody response directed against several linear epitopes which seems to mimic the antibody response in PUUV infection [145].

Alternatively, *Alphavirus* (Sindbis virus) replicons have been evaluated as new potential *Hantavirus* vaccine vectors. However, both DNA-based Sindbis replicon and packaged Sindbis replicon vectors containing either the M or S genome segment of SEOV only induced a protective immune response in some hamsters [129] (see *table II*).

3.5. Antiviral drugs

The only antiviral drug in use against *Hantavirus* infections is ribavirin. Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been described as a broad-spectrum antiviral agent (for a review see [146]). Ribavirin has been demonstrated to be effective against a Vero E6-passaged human HTNV isolate in vitro [147] and reduced viraemia and case fatality in experimentally HTNV-infected suckling mice [148]. A prospective, randomised, double-blind, concurrent and placebo-controlled clinical trial of intravenous ribavirin in 242 confirmed HFRS patients in the People's Republic of China revealed a significant reduction in fatality and in the risk of entering the oligouric phase and of experiencing haemorrhage. The only adverse effect of the ribavirin treatment was the development of a well-recognised anaemia which was completely reversible after completion of the therapy [149]. In contrast, a recent study on HPS patients did not suggest an appreciable drug effect [150].

Pretreatment of Vero E6 cells with human interferons resulted in a dose-dependent inhibition of HTNV replication. In line with these data mouse IFN-β inhibited the replication of HTNV in murine macrophages and increased the survival rate of HTNV-infected newborn mice [151]. Similar effects were observed with PUUV. The antiviral effect of interferon is mediated by the induction of the antiviral MxA protein [152]. Stably MxA-transfected Vero

E6 cells were able to inhibit both RNA and viral protein accumulation of PUUV, TULV [153] and HTNV [154].

Other efforts to study directly the inhibition of the *Hantavirus* replication by nucleoside analogues or non-nucleosidic inhibitors will require the development of in vitro transcription/replication systems or of cells stably expressing viral RNA polymerase. There is as yet no structural information about the polymerase available which would allow a rational structure-based antiviral drug design. Other antiviral strategies proven to be successful in vitro for other RNA viruses, such as the use of antisense nucleic acid sequences, ribozymes or inhibitors of uncoating or assembly, have not been established for the inhibition of *Hantavirus* replication.

4. Outlook

In the future, one can expect extended knowledge on the geographical distribution of the various hantaviruses, an improvement in the diagnosis of *Hantavirus* infections and more awareness of HFRS and HPS by physicians and the public. A better understanding of those components of immune response being important for protection against *Hantavirus* infections should enable the design of potent vaccines. The progress should be accelerated by the establishment and exploitation of a useful animal model developing the clinical signs of infection. Human vaccines should be applied to groups at risk from *Hantavirus* infections, as well as inhabitants of epidemiologically high-risk areas or people at risk through their profession.

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