Hantavirus infections in The Netherlands

Hantavirusinfecties in Nederland

Proefschrift

Ter verkrijging van de graad van doctor

aan de Erasmus Universiteit Rotterdam

op gezag van de Rector Magnificus

Prof. Dr. P.W.C. Akkermans M.A.

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 10 januari 1996 om 11.45 uur

door

Jan Groen

geboren te Amersfoort

Promotie commissie

Promotor: Prof. Dr. A.D.M.E. Osterhaus

Overige leden: Prof. Dr. J. van der Noordaa
Prof. Dr. W. Weimar
Prof. Dr. G. van der Groen

CIP GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Jan Groen

Hantavirus infections in The Netherlands / Jan Groen
Rotterdam: Erasmus Universiteit Rotterdam
- Met lit. opg. - Met samenvatting in het Nederlands.
ISBN 90-9008981-0
Trefw.: HFRS / NE / Hantavirus

The studies described in this thesis were conducted at the former Laboratory of Immunobiology (Head Prof. Dr. A.D.M.E. Osterhaus) of the National Institute of Public Health and Environmental Protection, Bilthoven, and at the Department of Virology (Head Prof. Dr. A.D.M.E. Osterhaus), University Hospital Rotterdam, The Netherlands.

The work was supported by the National Institute of Public Health and Environmental Protection (RIVM) in Bilthoven, Veterinary Public Health Inspectorate (VHI) and the Dutch Ministry of Defense.

Printing of this thesis was financially supported by: **Techno-Lab International, Alkmaar**; MP-Products, Soest; European Veterinary Laboratory, Woerden; Merlin Diagnostic Systems, Rotterdam; Uniprom Diagnostics, Waddinxveen and BioMérieux, 's-Hertogenbosch The Netherlands.

Vormgeving: Charlotte Groen-Burgmans
Drukwerk: Febodruk B.V., Enschede

To my family

Contents

Abbreviations

ALAT	alanine aminotransferase
BSA	bovine serum albumin
CFA	complement fixation assay
CTB-ELISA	complex trapping blocking - enzyme linked immunosorbent assay
DMSO	dimethylsulfoxide
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
G protein	glycoprotein
GMV	geometric mean value
HDPA	high density particle assay
HFRS	haemorrhagic fever with renal syndrome
HIA	haemagglutination inhibition assay
HPS	hantavirus pulmonary syndrome
HRPO	horse-radish-peroxidase
HV	Hantavirus
HVD	hantaan virus disease
IFA	immunofluorescence assay
Ig	immunoglobuline
kD	kilodalton
LB	lyme borreliosis
LCMV	lymphocytic choriomeningitis virus
MCA	monoclonal antibody
MS	mean score
Mw	molecular weight
NE	nephropathia epidemica
NP	nucleoprotein
OD	optical density
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PRNT	plaque reduction neutralization assay
PUUV	Puumala virus
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SPF	specific pathogen free
TBE	tick-borne encephalitis virus
TMB	tetramethyl benzidine
WHO	World Health Organization

CHAPTER 1 2 3 4 5

General Introduction

Partly taken from:

Infectie met hantavirus, een te weinig herkende oorzaak van acute nierinsufficiëntie
H.G.M. Jordans, J. Groen, J. Clement and A.D.M.E. Osterhaus
Nederlands Tijdschrift voor Geneeskunde 1991, 18:791-793

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Epidemiology and laboratory diagnosis of hantavirus (HTV) infections
J.P. Clement, P. McKenna, J. Groen, A.D.M.E. Osterhaus, P. Colson, T. Vervoort, G. Van der Groen and H.W. Lee
Acta Clinica Belgica 1995, 50:9-19

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M.N. Gerding, J. Groen, J.G.M. Jordans and A.D.M.E. Osterhaus
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Nederlands Tijdschrift voor Medische Microbiologie 1995, 4:6-10

History

During the Korean war between 1951-1953, an epidemic occurred among United Nations sanctioned military troops, characterized by fever, haemorrhages, shock and renal failure with a case fatality rate of up to 15%. This disease became known as Korean haemorrhagic fever [Smadel, 1951; Earle, 1954]. Despite extensive investigations at that time no etiologic agent could be identified. In the far east the oldest suggestive description of a similar disease had been made as early as 1000 years ago in a Chinese medicine book [Lee, 1982a]. During the American Civil War between 1862-1866 epidemics of "general dropsy" with renal involvement were noted among northern army troops. This disease was later called "Bright's disease" [Brown, 1916]. In Europe the first descriptions of the disease appeared in the early 1900's, after outbreaks in Russia and Scandinavia [Mayer, 1952; Myhrman, 1934; Zetterholm, 1934]. The Russians called the disease haemorrhagic nephroso-nephritis and the Scandinavians named it nephropathia epidemica (NE). During the trench-warfare of World War I in Europe, several epidemics of NE-like disease occurred among British, French and German troops. In the army medical records of the respective countries this "newly" described disease became known as "trench-nephritis", "nephrite de guerre" and "Kriegsnephritis" [Brown, 1916; Ameuille, 1916; Ullman, 1916]. In World War II several thousands of cases of "Kriegsnephritis" occurred among German troops in northern Finland [Stuhlfauth, 1943; Hortling, 1946]. Gajdusek postulated already in 1962 that all the above mentioned diseases were caused by a single agent or a closely related group of agents [Gajdusek, 1962]. In 1978, more than 25 years after the end of the Korean war, Lee and co-workers isolated the etiologic agent of Korean haemorrhagic fever, - Hantaan virus - from the lungs of Korean striped field mice (*Apodemus agrarius*) [Lee et al., 1978]. Outside Scandinavia and east Asia the first serologically confirmed human cases of Hantaan-like virus infection were described in Belgium in 1983 [Desmyter et al., 1983]. Subsequently, it has indeed become clear that all the above mentioned diseases are caused by closely related viruses [Gajdusek, 1982; Lee, 1989] which have now been included in the genus *Hantavirus* of the *Bunyaviridae* family. In 1983, the rodent-borne viruses serologically related to Hantaan virus, all causing similar clinical symptoms, were labelled under the generic term haemorrhagic fever with renal syndrome (HFRS) [WHO, 1983].

The virus

Hantavirus particles are spherical enveloped viruses with peplomers arranged in a

distinct pattern, with a diameter of 100-120 nm and a tripartite RNA genome of negative polarity. The virus has a buoyant density in sucrose of 1.16-1.17 g/ml and in cesium chloride 1.20-1.21 g/ml [Schmaljohn and Dalrymple, 1983]. The RNA genome has a total Mw of about 4.5 x 10^6 D, and consists of a large (L), a medium (M) and a small (S) segment of about 6,500, 3,700 and 1,800 nucleotides respectively. All hantaviruses possess the same 3'-terminal nucleotide sequence (5'AUC.AUC.AUC.UG3') on each of their three RNA segments. The L genomic segment presumably encodes the virion-associated polymerase (Mw of about 200 kD). The M segment has one single, open reading frame. This frame contains two non-overlapping genes encoding the two envelope glycoproteins, the G1 (Mw of 65 kD to 74 kD) and G2 (Mw of 55 kD to 65 kD) glycoproteins. Both envelope glycoproteins show haemagglutinating activity [Tsai et al., 1984]. The S-segment also has a single, open reading frame, encoding the viral nucleoprotein (Mw of 50 kD to 53 kD) [Schmaljohn et al., 1986; Parrington and Kang, 1990; Arikawa et al., 1990]. The nucleoprotein is the most conserved protein among the different hantavirus subtypes [Sheshberaraden et al., 1988].

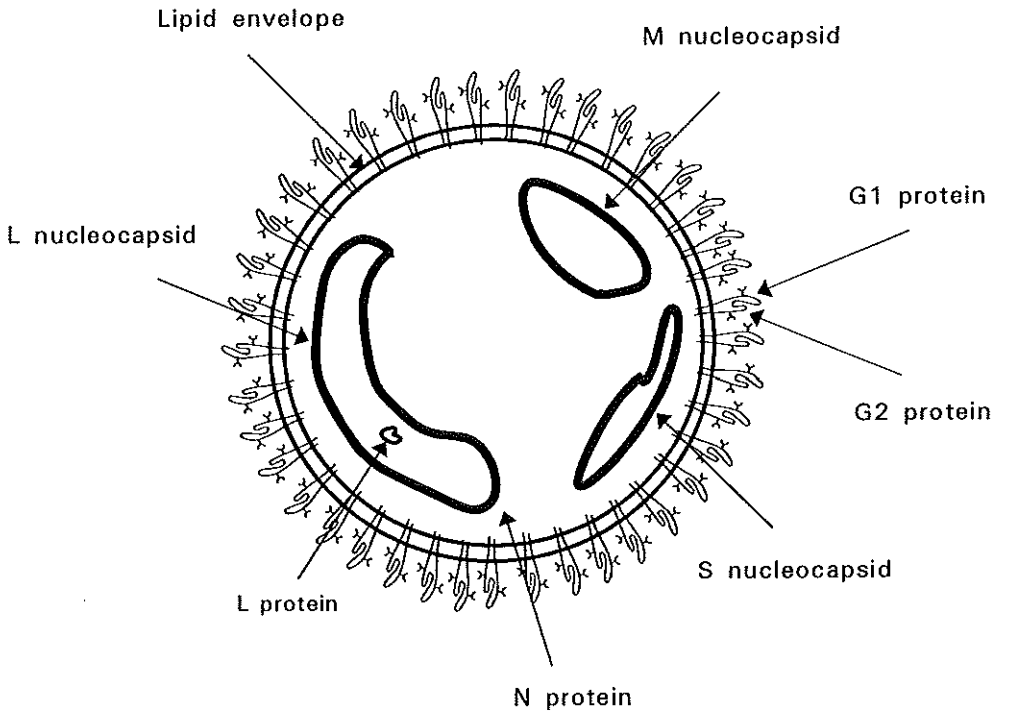

Figure 1. Schematic representation of a hantavirus particle.

Hantaviruses are sensitive to deoxycholate and lipid solvents (chloroform, ether, acetone), relatively stable at 4°C-20°C, and are inactivated rapidly at temperatures above 37°C and at pH values below 5. On basis of sequence analyses all hantaviruses have been shown to be genetically related [Bishop, 1990; Chu et al., 1994].

Classification of hantaviruses

In 1987, the International Committee on Taxonomy of Viruses incorporated Hantaan virus as the prototype virus, together with a number of related viruses into the new genus *Hantavirus* of the family *Bunyaviridae*. This was based on antigenic, morphological, physical and genomic properties. The establishment of a new genus was based on the absence of serological cross-reactivity with any of the other members of the *Bunyaviridae* family, a unique sequence of conserved terminal nucleotides and gene segment coding strategies, which differed from the other genera of this family. Hantaan virus (strain 76-118) was named after the river Hantaan in Korea [Lee, 1982a]. In contrast to members of the other genera of the *Bunyaviridae*, members of the genus *Hantavirus* have not been recovered from arthropods, and most isolates have been derived from rodent species. Hantavirus specific serum antibodies and antigens have been detected in a large number of rodent species, but also in other mammalian and avian species from more than 35 countries all over the world [Tkachenko and Lee, 1991; Clement et al., 1994a]. Recently, two new isolates have been defined as candidates for new subtypes. The first is an isolate from the bandicoot (*Bandico indica*) in Thailand with unknown pathogenicity for humans [Xiao et al., 1992]. The second is Sin Nombre virus, which was isolated from the deer mouse (*Peromyscus maniculatus*) in the USA and identified as the causative agent of a severe and often fatal respiratory disease in man [Elliott et al., 1994].

The severity of HFRS caused by the members of the genus Hantavirus largely depends on the subtype of the virus involved. So far eight different subtypes of this genus *Hantavirus* have been identified (Table 1) [Lee et al., 1990a; Nichol et al., 1993]. The proposed subtypes are closely associated with the genus of their reservoir host (Table 1). Hantaviruses are transmitted to man and other species by direct or indirect contact with persistently and subclinically infected rodents which serve as their reservoir. The most important rodent carriers of hantaviruses distributed all over the world are: *Apodemus, Rattus, Clethrionomys and Peromyscus* species. Hantaan-like virus infections are mainly associated with *Apodemus* species and predominantly found in South East Asia and Central Europe. Seoul-like virus

infections associated with *Rattus* species are mainly found in harbour- and urban areas all over the world. Infections with Puumala virus are associated with *Clethrionomys* species and found in Scandinavian- and other European countries. *Peromyscus* species are the predominant carriers of the newly discovered Sin Nombre virus and so far only found in the United States.

Nucleotide sequence comparison of the S segment of Puumala virus with that of Hantaan virus, Seoul virus (SR-11) and Prospect-Hill virus showed homologies of 60.0%, 60,5% and 69.2%, respectively, and comparison of the M-segment showed homologies of 59.0%, 58.3% and 70.0% respectively [Antic et al., 1992; Vapalahti et al., 1992]. The overall nucleotide sequence similarity between the respective subtypes of the genus *Hantavirus* is approximately 70% [Nichol et al., 1993; Xiao et al., 1993].

Table 1. Hantavirus subtypes with their primary mammalian host and associated human disease.

Virus	Host species	Disease in humans	Reference
Hantaan	Striped field mouse (*Apodemus agrarius*)	severe HFRS[1]	Lee, 1978
Seoul	Norway rat (*Rattus norvegicus*)	moderate HFRS	Lee, 1982b
Dobrava	Yellow necked field mouse (*Apodemus flavicollis*)	severe HFRS	Avsic-Zupanc, 1992
Puumala	Red bank vole (*Clethrionomys glareolus*)	NE[2]	Niklasson, 1984
Sin Nombre	Deer mouse (*Peromyscus maniculatus*)	HPS[3]	Elliott, 1994
Prospect Hill	Meadow vole (*Microtus pennsylvanicus*)	unknown	Lee, 1982
Thailand	Badicoot (*Badicota indica*)	unknown	Xiao, 1992
Thottapalayam	Shrew (*Suncus murinus*)	unknown	Karabatsos, 1985

[1]HFRS :Haemorrhagic fever with renal syndrome
[2]NE :Nephropathia epidemica
[3]HPS :Hantavirus pulmonary syndrome

Virus replication

In contrast to the members of three other negative-sense RNA virus families (*Orthomyxo-*, *Paramyxo-*, and *Rhabdoviridae*), members of the *Bunyaviridae* lack an

internal matrix protein. Therefore, the process of viral morphogenesis must be substantially different. Replication of hantaviruses takes place in the cytoplasm of the host-cell [Petterson, 1991]. The virus enters the host-cell by fusion of the virus-envelope with the cell membrane probably by mechanisms mediated by a specific receptor that has not yet been identified. *In vitro* studies demonstrated cell fusion at low pH [Arikawa et al., 1985], but it is unknown if the fusogenic property resides in the glycoprotein G1 or G2. The haemagglutinating activity of the glycoproteins of hantaviruses may be inhibited by monoclonal antibodies. However, not all of these monoclonal antibodies neutralize virus infectivity. The viral genome is first transcribed into mRNA by the virion-associated RNA polymerase. Synthesis of the viral polypeptides shortly after infection suggest that mRNAs are transcribed and translated rapidly. Maturation of virus particles occurs in the intracellular smooth membranes of the Golgi apparatus, which is morphologically parallelled by a progressive vacuolization of the infected cells. Subsequently, virions are transported to the cell membrane, where exocytosis takes place to release the virus [Elliott, 1990].

Pathogenesis of hantavirus infections

The pathogenesis of hantavirus infections differs between the different host species. The virus is generally non-pathogenic for rodents: with the exception of experimentally infected new-borne mice and rats [Yamanouchi et al., 1984], rodents remain persistently infected without showing any clinical signs [Zhu et al., 1986]. Persistently infected rodents excrete the virus via saliva, urine and lungs [Tsai., 1987a]. Virus and viral antigen have been demonstrated in virtually all organs and organ systems of rodents, but most prevalent in lungs, nerve tissue and vascular endothelium.

The pathogenesis of hantavirus infections in humans is poorly understood. Most of the pathological findings in humans are based on biopsy and autopsy specimens [Collan et al., 1991; Hung et al., 1992]. The acute phase of the infection and prodromal changes, are usually not recognised as being caused by a hantavirus infection. Therefore, studies of the pathogenesis of this phase of infection have largely been limited to studies in animal models. Mice experimentally infected with Hantaan virus demonstrated that cell-mediated immunity rather than humoral immunity plays an important role in the protection against fatal disease [Nakamura et al., 1985; Yoshimatsu et al., 1993]. This was also supported by showing that rodents vaccinated with recombinant nucleoprotein, which does not induce virus neutralizing antibodies, were protected against infection [Schmaljohn et al., 1990].

The role of cell-mediated immunity in humans was demonstrated by comparing the peripheral blood mononuclear cells (PBMC) of serologically confirmed HFRS patients during the acute and convalescent phase. It was shown that in the acute phase T lymphocytes count and CD4:CD8 ratios decrease whereas the B lymphocyte counts and interferon-gamma expression on PBMC surfaces increase. At the same time, the ratio between activated antigen positive lymphocytes and PBMC in the acute phase was higher than that in the convalescent phase [Huang et al., 1994].

Several observations suggest that the direct cytopathic effect of the virus is an important factor in the pathogenesis of HFRS [Cosgriff, 1991]. Viral antigen has been detected in vascular endothelium cells in autopsy specimens of HFRS patients [Grcevska et al., 1990; Cosgriff, 1991] and in *in vitro* infected human vascular endothelial cells inoculated with different virus strains [Yanagihara and Silverman, 1990]. This indicates that direct effects of viral cytopathogenicity may play an important role in the vascular dysfunction. This may lead to increased permeability as manifested by focal haemorrhages [Cosgriff, 1991; Pensiero et al., 1992]. Local effects of vascular dysfunction on renal vessels, and dysfunction of renal tubular epithelial cells may be important factors in renal failure [Cosgriff, 1989]. The histopathological findings in kidney biopsies from these patients vary according to the time elapsed after the onset of the illness [Collan et al., 1991]. The histopathological damage in the interstitium, tubule and glomerulus is relatively mild in HFRS and NE patients. The most important histological findings in the kidneys of these patients are medullary haemorrhages, edema and tubular abnormalities like sloughing and necrosis of tubular epithelium [Collan et al., 1991; Mustonen et al., 1994]. During infection the kidneys of these patients appear to be swollen. It has been suggested that immune mediated mechanisms account for at least part of the pathogenesis of hantavirus disease. These include the formation of immune complexes and the activation of the classical complement pathway [Yan et al., 1981; Penttinen et al., 1981; Cosgriff, 1991].

Clinical aspects

Three clinical presentations of HFRS have been described which are associated with the different subtypes of the genus *Hantavirus*. These forms are severe HFRS and moderate forms of HFRS and hantavirus nephropathy [Tsai, 1987b; Childs and Rollin, 1994]. Recently, another clinical presentation of a hantavirus infection has been described, which was caused by the newly identified Sin Nombre virus. Infection with this virus proved not to be associated with HFRS, but with severe respiratory disease in humans in the United States [Duchin et al., 1994].

Table 1. Comparison of the clinical symptoms and laboratory findings in humans with different forms of hantavirus infection.

Virus	Hantaan	Seoul	Puumala	Sin Nombre
Clinical symptoms	(Severe HFRS)	(moderate HFRS)	(NE)	(HPS)
Fever	■	■	■	■
Malaise	■	■	■	■
Back pain	■	■	■	
Abdominal pain	■	▒	▒	▒
Headache	▒	▒	▒	■
Nausea/vomiting	▒	▓	▒	▒
Polyuria	■	■	▒	
Anuria	■	■	▒	
Myopia	▓	▓	▓	
Haemorrhage	■	▓	▓	
Hypotension	▒	▓	▓	▒
Shock	▒	▓		▒
Lung infiltrates	▓		▓	■
Abnormal respiratory rate				■
Shortness of breath				■
Laboratory findings				
ASAT/ALAT ↑	▒	▓	▓	▒
Lactate dehydrogenase ↑	▓	▓	▓	■
Thrombocytopenia	▒	▒	▒	▒
Serum creatinine ↑	■	■	■	▓
Proteinuria	■	■	▒	
Haematuria	■	■	▓	▓
Haemoconcentration	▒	▒	▓	▒
Polyleukocytosis	▒	▓	▒	▒

■ 100-90% ▒ 90-50%; ▓ 50-10%; ▓ 10-1%; of patients

Severe forms of HFRS are caused by Hantaan-like viruses with case fatality rates between 5% and 15% [Tsai., 1987b; Childs and Rollin, 1994]. This severe disease can be divided into five subsequent characteristic phases: the febrile, the hypotensive, the oliguric, the diuretic and the convalescent phase. The febrile phase starts with a sudden onset of high fever, chills, headache, malaise, backache and orbital pain. In most of the cases massive proteinuria and microhaematuria appear suddenly toward the end of the febrile phase. Development of the hypotensive phase, lasting from several hours to some days, is characterized by conjunctival haemorrhages and fine petechial rash which commonly develops on the face, upper thorax, axillae, and legs. This phase may lead to shock and death. Haemodynamic alterations appear to be the direct consequence of vascular dysfunction and may lead to hypotension and shock. The oliguric phase lasts from three to seven days and is marked by nausea, vomiting, decrease in urinary output and increased blood urea and serum creatinine. Clinical recovery usually starts during the diuretic phase, is associated with the improvement of renal function and may last from a couple of days to several weeks. The fast majority of the patients recovers completely during the convalescent phase.

The clinical presentation of moderate HFRS caused by Seoul-like virus strains is largely similar to that of Hantaan-like virus infections, but the symptoms are less severe and the case fatality rate is less than 1% [Tsai, 1987b; Lee and Van der Groen, 1989; Lee et al., 1990a; Childs and Rollin, 1994].

The mild form of HFRS, caused by Puumala-like viruses which is known as NE, shows similarities with HFRS, but clinical symptoms are more variable and often difficult to define as belonging to this disease entity (Table 2) [Pether and Lloyd, 1993]. Especially the haemorrhagic manifestations are rarely seen and the case fatality rate in untreated cases is low (< 0.5%) [Tsai, 1987b; Childs and Rollin, 1994]. Infection with Puumala virus is characterized by a biphasic course: the first phase is associated with an influenza-like illness (fever, malaise, chills, lumber pain) lasting for four to ten days, and the second phase is associated with acute transient renal failure. During this phase, oliguria may occur, followed by a short and massive proteinuria and microhaematuria. Within two to three weeks patients recover spontaneously with a normal kidney function [Tsai, 1987b; Van Ypersele de Strihou and Mery, 1989; Settergren et al., 1989].

In contrast to HFRS associated viruses, the target organ of the recently identified Sin Nombre virus in humans seems to be the respiratory tract rather than the kidney. This newly recognised presentation form, known as hantavirus pulmonary syndrome (HPS) is also characterised by a non-specific prodromal stage with fever, headache, myalgia, nausea and conjunctivitis, accompanied by respiratory signs like coughing and dyspnea, which may lead to a sudden respiratory failure within several hours or days (Table 2). So far, infection with this virus has the highest case fatality rate

(>60%) amongst the known hantaviruses [Childs and Rollin, 1994; Duchin et al., 1994].

Laboratory diagnosis

Although several laboratory techniques can be used to diagnose hantavirus infections, the diagnosis is usually made by the demonstration of hantavirus-specific antibodies or antigen. Virus isolation from clinical specimens is difficult, time consuming and hazardous to perform [Yao et al., 1989; Xiao et al., 1991; Grankvist et al., 1993]. Propagation of the virus requires special P3 facilities [McCormick et al., 1982; Saluzzo et al., 1988]. Most hantaviruses can be propagated in Vero E6 cells, but also other cell types are permissive [Temonen et al., 1993]. Several serological techniques have been described for the detection of hantavirus specific antibodies, including immunofluorescence assays (IFA), haemagglutination inhibition assays (HIA), complement fixation assay (CFA), plaque reduction neutralization tests (PRNT), enzyme-linked immunosorbent assays (ELISA), high density particle agglutination (HDPA) tests and Western-blot analyses [Tkachenko et al., 1981; Sugiyama et al., 1984; Goldgaber et al., 1985; Lee et al., 1985; Takenaka et al., 1985; Okuno et al., 1986; Dantes et al., 1987; Zöller et al., 1989; Tomiyama and Lee 1990; Tang et al., 1991].

The IFA has been used extensively for the serodiagnosis of hantavirus infections, screening of animals and selected human populations, and is still the most widely used assay. Besides the detection of specific Ig antibodies in serum, IFA can also be used to provide an indication for the hantavirus subtype involved by comparing serum antibody titers against the respective hantavirus antigens. Also other serological assays like the HIA and ELISA can be used for the tentative serological identification of infecting hantavirus subtypes [Goldgaber et al., 1985; Tang et al., 1991]. However, more reliable results are obtained by using the PRNT [Lee et al., 1985; Niklasson et al., 1991]. As such, the demonstration of serum antibodies to hantavirus only allows the identification of individuals who have experienced a hantavirus infection in the past, without the possibility to estimate the time elapsed after infection.

For the detection of hantavirus specific IgM or a rise of IgG serum antibodies, IFA or ELISA systems are often used to confirm the clinical diagnosis of an acute hantavirus infection. However, it has been shown that IgM antibodies may, in rare cases, persist for long periods after infection [Ivanov et al., 1988; Niklasson et al., 1990] and it has also been demonstrated that hantavirus infected patients may already have relatively high IgG titers in the acute phase of the illness [Hedman et

al., 1991; Lundkvist et al., 1993]. Furthermore, paired serum samples are often not available. The recently described IgG specific avidity-IFA allows estimation of the time elapsed after the onset of illness [Hedman et al., 1991; Kallio-Kokko et al., 1993].

Although for most of the above mentioned assays gamma irradiated or inactivated hantavirus antigens can be used, the production of these antigens still requires P3 facilities [Saluzzo et al., 1988]. More recently, recombinant hantavirus nucleoprotein and the glycoproteins G1 and G2 have been expressed by recombinant vacciniaviruses, baculoviruses and E-coli systems. These recombinants proved to provide good alternative sources of antigen for the development of hantavirus specific diagnostic assays [Schmaljohn et al., 1988; Rossi et al., 1990; Gott et al., 1991; Wang et al., 1993; Yoshimatsu et al., 1993; Zöller et al., 1993].

The difficulty to isolate hantaviruses from clinical specimens, the pathogenicity of hantaviruses to humans and the slow replication rate *in vitro* prompted the development of more rapid, sensitive and safe detection systems for these viruses. In 1991, Xiao and coworkers demonstrated that the sensitivity of a reverse transcriptase polymerase chain reaction (RT-PCR) was equal to virus isolation from mice, experimentally infected with Hantaan virus. Several other studies used RT-PCR for *in vitro* detection, and also for subtyping of hantavirus isolates [Giebel et al., 1990; Tang et al., 1990; Puthavathana et al., 1992]. In cells from the urinary-, and respiratory tracts and in PBMC from humans with NE, viral RNA was detected using a nested RT-PCR [Grankvist et al., 1992]. Recently RT-PCR proved to be an important tool for the identification of the cause of the outbreak of HPS in humans in the South Western United States [Nichol et al., 1993]. In 1994, the presence of a hantavirus named Tula virus amongst European common voles was demonstrated by PCR, and subsequent sequence analysis [Plyusnin et al., 1994] showed that this virus was different from all previously identified hantaviruses. Thus, molecular techniques have also become important diagnostic tools for hantavirus detection and identification. These techniques have recently allowed studies of the genetic drift of these viruses in their respective host populations [Plyusnin et al., 1994].

Epidemiology

The hantaviruses that cause the zoonotic disease HFRS occur in geographical foci. Epidemic outbreaks do occur, but most of the HFRS cases are sporadic. These outbreaks are associated with the surges in their reservoir rodent populations related to climatological or other environmental changes [LeDuc, 1987; Yanagihara and Gajdusek, 1988]. Epidemiological studies based on clinical descriptions of this disease started after the first documented human cases. After the isolation of

Hantaan virus from the Korean striped field mouse in 1976 [Lee et al., 1978], several serological techniques using Hantaan virus (strain 76-118) as antigen have been used in seroepidemiological surveys among humans and animals. They demonstrated a wide distribution of hantavirus infection all over the world (Figure 2) [Tkachenko and Lee, 1991]. Hantavirus subtype-specific assays demonstrated that severe HFRS caused by Hantaan-like viruses including Dobrava virus is predominantly found in South East Asia and Central Europe, Seoul-like virus infections causing moderate HFRS are distributed world-wide, Puumala-like virus infections causing NE are mainly found in Europe (Figure 3) [Antoniadis et al., 1987; Sommer et al., 1988; Filipe et al., 1991; Tkachenko and Lee, 1991; Pether and Lloyd, 1993; Ahlm et al., 1994; Le Guenno et al., 1994] and so far Sin Nombre virus associated with severe HPS has only been found in the USA [Childs and Rollin, 1994; Elliott et al., 1994].

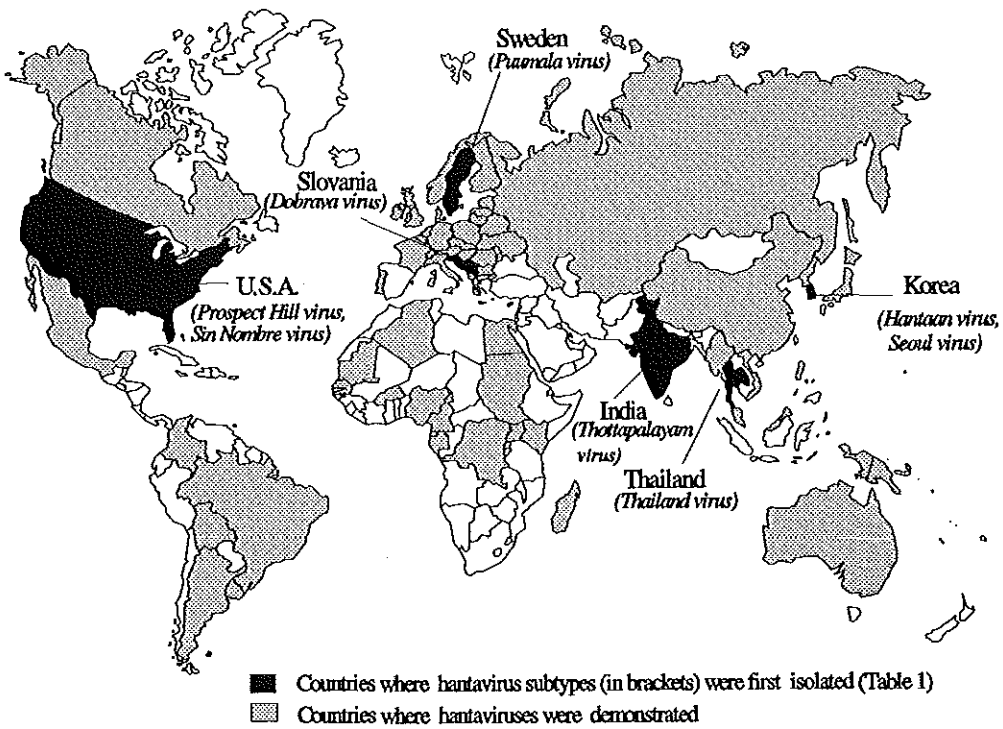

Figure 2. Worldwide distribution of hantavirus.

In 1984, the first cases of hantavirus infections in The Netherlands were documented among laboratory workers, who had been in contact with infected laboratory rats. Subsequently, a number of non-laboratory associated cases were

reported at different locations in The Netherlands [Osterhaus et al., 1984]. In the same year the causative agent of NE was isolated from red bank voles in Sweden [Niklasson and LeDuc, 1984; Yanagihara et al., 1984].

The incidence rates of hantavirus infections vary between different endemic regions and between seasons. In the People's Republic of China the annual incidence ranged from 30-168 cases per 100,000 individuals. Similar rates have been reported

Figure 3. Distribution of hantavirus subtypes in Western Europe: ⊡ Puumala-like virus identified; ▩ Hantaan/Seoul-like virus identified; ▲ Laboratory associated infections with Seoul-like virus.

in certain areas of the European part of Russia [Tkachenko and Lee, 1991], whereas in Europe and Scandinavia only a few hundred cases of NE are reported yearly [Niklasson et al., 1987; Sommer et al., 1988; Pilaski et al., 1991; Ahlm et al., 1994; Clement et al., 1994b; Le Guenno et al., 1994]. Infections with hantaviruses occur throughout the year but a certain seasonal pattern is observed [Yanagihara and Gajdusek, 1988]. In South East Asia, two seasonal peaks of HFRS cases occur in the spring and the summer, which correlate with the planting and harvesting seasons respectively [LeDuc, 1987]. In the Scandinavian countries, the majority of NE cases occurs during autumn and winter when rodents tend to seek human shelters [LeDuc,

1987]. In Western European countries NE cases occur throughout the year with a slight increase in the summer months. This is probably due to mild climatological conditions, which allow the reservoir rodents to survive outside a direct human environment.

Numerous epidemiological studies suggest that a close contact with rodents or rodent environments constitutes a certain risk for the acquisition of hantavirus infections. Compared to matched control groups, significantly higher percentages of hantavirus-specific antibodies have been found amongst individuals with an outdoor occupation, like farmers, hunters, soldiers, forestry workers, biologists, and animal trappers [Yanagihara, 1990]. Higher rates of hantavirus specific antibodies have also been found in patients with chronic renal failure, haemodialysis patients, and renal transplant candidates [Glass et al., 1990, 1993; Tsianos et al., 1993]. Furthermore, hantavirus infections are more often found in males than in females with a peak between 20 to 50 years of age [Tkachenko and Lee, 1991].

Control of hantavirus infections

Cases of hantavirus disease among laboratory workers handling laboratory rodents [Lee and Van der Groen, 1989] stressed the need for adequate virus screening programmes for laboratory animals. Since hantaviruses are not transmitted vertically in rodents, hantavirus free stocks of laboratory animals can be produced by caesarean section [McKenna et al., 1992]. Although prevention of hantavirus infections for certain occupations at risk, especially in known endemic areas, is difficult to achieve, knowledge of risk factors and rodent control may play a role in reducing the number of these infections.

Therapy for HFRS and NE patients is almost exclusively based on the symptomatic treatment. Haemodialysis has been shown to reduce mortality during the oliguric phase [Cosgriff, 1989]. Further reduction of mortality has been achieved by the use of antiviral therapy with Ribavirin® [Huang et al., 1988; Huggins, 1989]. Treatment of patients suffering from acute HFRS with interferon proved successful [Gui et al., 1987].

Since each year more than 100,000 cases of severe HFRS are reported in South East Asia, and no specific therapy is available at present, the development of a preventive vaccine is of major importance. A number of candidate vaccines have been developed, including inactivated Hantaan virus produced in rodent brains or cell culture, and recombinant vaccines [Lee et al., 1990b; Song et al., 1991, 1992; Schmaljohn et al., 1992; Yoshimatsu et al., 1993]. To date both mouse brain and cell culture vaccines have been used in humans, causing little side effects [Lee et al.,

1990b; Song et al., 1992]. However, their protective value remains to be shown. The development of a relevant non-rodent, animal model would offer the opportunity to test the value of hantavirus vaccines in challenge experiments.

Outline of the thesis

The aim of the studies described in this thesis is to develop and evaluate new diagnostic methods for hantavirus infections, to study their epidemiology in The Netherlands and to develop an animal model that will allow studies concerning the viral pathogenesis and induction of protective immunity against these infections. The studies presented are largely focussed on Puumala-like virus infections in The Netherlands.

Chapter 2 describes newly developed serological diagnostic methods for hantavirus infections. First, new ELISA systems are compared with the IFA which is still most commonly used for the detection of hantavirus-specific serum antibodies in humans. To create a more reliable system for the determination of the time elapsed after the onset of hantavirus infection and thus allow the diagnosis of acute infections, the development of the antibody response against the different structural proteins and the detection of different Puumala virus specific antibody classes and subclasses is studied at different times after infection. In addition, studies are presented in which biopsies from kidneys of patients with NE are examined for histopathological abnormalities and for the presence and localization of viral antigen.

Chapter 3 describes the screening of patient sera from The Netherlands using different IFA and ELISA systems for the presence of antibodies against different hantavirus subtypes. Serosurveys are presented which study the prevalence of hantavirus infections in The Netherlands amongst selected human populations with and without occupational risk for acquiring hantavirus infections, and amongst feral and domestic animal populations.

Chapter 4 describes the first non-human primate model for hantavirus infection, which can be used to study the pathogenesis, therapeutic approaches and immunization strategies against Puumala virus infection. The potential of monkeys to develop models for studying the pathogenesis of other hantaviruses is indicated.

Chapter 5 summarizes and discusses the data presented in this thesis, in the light of the management of hantavirus infections in The Netherlands and their impact on future studies concerning the pathogenesis, prevention and therapy.

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CHAPTER 1 2 3 4 5

Diagnosis of Hantavirus infections

Comparison of immunofluorescence and enzyme-linked immunosorbent assays for the serology of hantaan virus infections
J. Groen, G. van der Groen, G. Hoofd and A.D.M.E. Osterhaus
Journal of Virological Methods 1989, 23:195-203

Serum antibodies to structural proteins of hantavirus arise at different times after infection
J. Groen, J. Dalrymple, S. Fisher-Hoch, J. P. Clement and A.D.M.E. Osterhaus
Journal of Medical Virology 1992, 37:283-287

Class and subclass distribution of hantavirus-specific serum antibodies at different times after the onset of nephropathia epidemica
J. Groen, M. Gerding, H.G.M. Jordans, J.P. Clement and A.D.M.E. Osterhaus
Journal of Medical Virology 1994, 43:39-43

Histopathological findings and Hantavirus antigen detection in kidney biopsies from patients with nephropathia epidemica
J. Groen, J.A. Bruijn, M.N. Gerding, H.G.M. Jordans, A.W. Moll van Charante and A.D.M.E. Osterhaus
Clinical Nephrology 1995, submitted

Comparison of immunofluorescence and enzyme-linked immunosorbent assays for the serology of Hantaan virus infection

Jan Groen, Guido van der Groen, Guy Hoofd and Albert D.M.E. Osterhaus
Journal of Virological Methods 1989, 23:195-203

Summary

Three enzyme-linked immunosorbent assay (ELISA) systems based upon different principles were developed for the serology of Hantaan virus infections and compared with an indirect immunofluorescence assay (IFA). The indirect IFA was carried out with γ-irradiated Hantaan virus-infected and uninfected Vero E6 cells fixed with ethanol (-70°C) or acetone (20°C) on drop slides and a FITC-coupled sheep anti-human Ig preparation. Atypical staining in IFA was avoided by using ethanol (-70°C) instead of acetone (20°C) fixation. In the first ELISA ('cell-assay'), Hantaan virus-infected or uninfected Vero E6 cells were used as antigens, which after γ-irradiation were seeded into microtiter ELISA strips. Serial dilutions of human sera were incubated and specific antibodies were demonstrated with a horseradish peroxidase (HRPO)-conjugated sheep anti-human Ig preparation. In the second ELISA ('competition-assay') an affinity-purified human Ig preparation was used as a capture antibody for Hantaan virus antigen. After incubation of serial dilutions of human sera with this coat, the reactivity of the affinity purified anti-Hantaan virus Ig coupled to HRPO was determined. In the third ELISA ('complex trapping blocking [CTB]-assay') the same capture antibody was used to react with a mixture of the antigen and serial dilutions of human sera.

The reactivity with the same HRPO conjugate was then determined. The results obtained in the respective assay systems with sera from people at risk or suspected of Hantaan virus infection coincided well. The CTB-ELISA proved to be faster and more sensitive than both the other ELISA systems, without giving more non-specific reactions: it detected almost all the IFA positive samples. This was also confirmed by Western blotting analysis of sera showing discordant results in the respective assays. The sensitivity, specificity and rapidity of the CTB-ELISA makes it suitable for routine use for the serology of Hantaan virus infections of man and animals.

Introduction

In 1976, Lee et al. isolated Hantaan virus as the etiological agent of Korean hemorrhagic fever from the lungs of the striped field mouse (*Apodemus agrarius*) [Lee et al., 1978]. This virus was named after the Hantaan river in Korea and has been classified as the prototype of the genus Hantaan virus (HV) of the family *Bunyaviridae*. It is unquestionably the most widespread zoonotic pathogen recognised since the discovery of lymphocytic choriomeningitis virus (LCMV) [Johnson, 1986]. The virus may be transmitted by clinically health rodents to humans through direct or indirect contact. Apart from human infections by contacts with wild rodents, laboratory workers in many countries have been reported to have contracted Hantaan virus disease (HVD) through contact with laboratory rodents [Kawamata, 1983]. Hantaan viruses have also been isolated from rat immunocytomas, which were shown to retain their infectivity during transplantation and storage procedures [Lloyd and Jones, 1986].

Fast and reliable serological test procedures are needed for the demonstration of HV-specific antibodies, for medical diagnostic procedures, screening programmes of laboratory animal colonies and for the control of biological products produced or controlled in rodents or rodent cells. The screening of rat immunocytomas producing monoclonal antibodies considered for diagnostic or therapeutic use in humans, seems especially important.

Several assays for the detection of Hantaan virus-specific antibodies have been described [Lee et al., 1978; Van der Groen et al., 1985]: immunofluorescence assays (IFA), a hemagglutination inhibition test (HI) and different enzyme-linked immunosorbent assays (ELISA) [Okuno et al., 1986; Igarashi et al., 1981; Takahasi et al., 1986]. IFA has been used extensively for the serodiagnosis of HVD and the screening of laboratory animal colonies. The assay is relatively fast and gives relatively high titers. However, standardization is difficult to achieve, mainly due to the variable amounts of viral antigen present in HV-infected cells [Takahasi et al., 1986] Furthermore, for the testing of large numbers of serum samples IFA is still too laborintensive and an assay more suitable to automation, like an ELISA, would be preferable. In this paper we describe the evaluation of three newly developed ELISA systems for the serology of HV infection with a panel of serum samples from humans at risk or suspected of HV infection. The results were compared with those obtained in an IFA and discrepant results were analysed in a confirmatory Western blotting assay.

Materials and methods

Virus and antigen preparation

Hantaan virus, strain 76-118, was passaged on Vero E6 cells in 150 cm^2 monolayer cultures in Eagle's MEM, supplemented with antibiotics and 10% fetal calf serum (FCS) for outgrowth and 2% FCS for maintenance. The virus and control antigens used for competition-ELISA and CTB-ELISA were prepared from 76-118 virus-infected and uninfected monolayer cultures of Vero E6 cells. Eight days after infection medium was removed, the cells were washed with phosphate buffered saline, pH 7.4 (PBS) and solubilized in 2.5 ml detergent solution (1% Triton X-100, 0.5% sodium desoxycholate, 0.1% sodium dodecyl sulphate, 0.5 M sodium chloride and 0.015 M Tris-HCL, pH 7.2) per culture. The solubilized cultures were then sonicated twice for 15 s after 3 cycles of freezing and thawing. Cellular debris was removed by centrifugation at 3000 x g for 10 min. The supernatant was used as antigen after storage at -20^{0}C.

Serum samples

A total of 102 sera was collected between 1984 and 1988 from people at risk or suspected of HV infection. The majority of the sera were of Belgian origin.

Preparation of capture antibody and conjugate

IgG fractions of sera from a HV-IFA positive and a HV-IFA negative donor were isolated by ammonium sulphate precipitation (50% saturation, pH 7,4) and affinity-purification through a Protein-A column as recommended by the manufacturer (Pharmacia, Uppsala, Sweden). Both purified IgG fractions were used as capture antibodies in the competition- and CTB-ELISA systems described below. Purified IgG from the seropositive donor was labeled with horse radish peroxidase (HRPO) (Sigma type P-8375), following the procedure described by Wilson and Nakane [Nakane et al., 1974]. Optimal working dilutions of the respective reagents used in both ELISA systems were determined by checkerboard titrations. Aliquots of 0.1 ml of the capture antibody and conjugate preparations were stored at -20°C until use.

Indirect immunofluorescence assay (IFA)

An IFA was performed, essentially as described previously [Van der Groen et al., 1985]. Briefly, infected Vero E6 cells were collected and suspended in growth medium containing 10% (v/v) dimethylsulfoxide at a density of 3.5 x 10^6 cells/ml. After gamma irradiation (2.5 x 10^6 rad), the suspension was stored in 2 ml volumes at -70°C until use. After thawing, the cell suspension was centrifuged for 5 min at 580 x g, the cells were washed three times in PBS and resuspended in PBS containing 5% FCS. After mixing with an equal number of uninfected Vero E6 cells, this suspension

was seeded onto Teflon-coated drop slides (Wellcome®, Beckenham, U.K.) at a concentration of 6 x 10^6 cells/ml (20 µl/well), air-dried and fixed for more than 30 min with acetone (20°C) or 98% ethanol (-70°C). Serial 2-fold dilutions of human serum samples in PBS were applied for 30 min. The slides were washed three times in PBS and a sheep anti-human Ig preparation conjugated with fluorescein isothiocyanate (kindly supplied by Dr. J. Nagel, RIVM) was then applied. After washing three times, slides were air-dried and read in an epifluorescence microscope (Leitz®, Jena, G.D.R.) at a magnification of 200 times. Results were considered positive when a characteristic dot-like immunofluorescence pattern was observed in the cytoplasm of infected cells. A so-called 'atypical pattern' of immunofluorescence observed with certain sera, was characterized by a more diffuse pattern of cytoplasmic immunofluorescence which disappeared at serum dilutions ≤ 1:64.

Cell-ELISA

A Hantaan virus-infected or uninfected y-irradiated Vero E6 cell suspension (10^6 cells/ml), washed tree times with PBS, was seeded into flat-bottom microtiter ELISA strips (50µl/well) (cat. No. 4-68667, Nunc, Denmark). The strips were airdried, fixed with ethanol (-70°C) containing 5% (v/v) acetic acid and stored with fixative at -70°C until use. Before use the strips were air-dried again, washed with PBS containing 0.05% Tween 80 pH 7,2 (PBS-T) and incubated for 1 h at 37°C with 100 µl of a blocking buffer (PBS-T, supplemented with 0.3% gelatin, 0.3% bovine serum albumin, 10% FCS) per well. The strips were washed three times with PBS-T and 50 µl volumes of HRPO-conjugated sheep anti-human Ig conjugate preparation (Amersham, U.K.) were added for 1h at 37°C. After washing three times with PBS-T, 100 µl volumes of the substrate tetramethylbenzidine (0.01% TMB, 1.67% DMSO, 10 µl H_2O_2 in 0.11 M sodium acetate buffer, pH 5,5) were added, and the reaction was stopped after 10 min by adding 100 µl volumes of 2.0 M H_2SO_4. The optical densities (OD450) were measured spectrophotometrically at 450 nm in a Titertek Multiskan (Flow Labs, U.K.).

Competition-ELISA

Titertek immunoassay plates (Flow Laboratories U.K.) were coated for 18 h at 20°C in 0.1 M carbonate/bicarbonate buffer (pH 9.6) with 100 µl volumes of the affinity purified human IgG preparation as capture antibody for Hantaan virus antigen. After washing the plates with H_2O containing 0.05% Tween 80, 100 µl volumes of PBS-T, supplemented with 0.5 M NaCl and 1% BSA, (PBS-TNB) were added, the plates were incubated for 30 min at 37°C and washed another three times. Two hundred microliter volumes of viral or control antigens were added to each well and incubated for 60 min at 37°C. The optimal antigen concentration had been determined by checker board titration and was defined as the highest concentration of

antigen giving an OD 450 of 0.8. Serial 2-fold dilutions of serum samples in PBS-TNB were added, the plates were incubated for 2 h at 37°C, washed three times and incubated for 2 h at 37°C with 100 µl volumes of the human affinity purified anti-HV IgG preparation coupled to HRPO. The plates were then washed three times and developed as described above.

Complex-trapping-blocking-ELISA (CTB-ELISA)

The CTB-ELISA was carried out essentially in the same way as the competition-ELISA. Instead of sequentially adding the antigen and serum dilutions, 50 µl volumes of serial diluents of serum sample were added together with 50 µl of antigen and incubated for 18 h at 20°C. After washing as described above, the conjugate was added and the plates were further processed as above.

Definition of ELISA antibody titers

All sera in the competition-ELISA, CTB-ELISA and cell-ELISA were tested against Hantaan virus control antigens and OD 450 values, measured against control antigen, were subtracted from OD values measured against viral antigen. These 'corrected' OD 450 values competition-ELISA and CTB-ELISA were considered positive if there was a reduction of 50% or more of the OD450 obtained with HV-IFA negative human sera. The 'corrected' OD450 values were considered positive if they exceeded the mean value plus three times standard deviation obtained with HV-IFA negative human sera. ELISA titers were expressed as the reciprocal of the highest serum dilution giving the indicated values in the respective ELISA systems.

Western blotting analysis

Western blotting analysis was performed as described by Franko et al. [1983]. The assay was considered positive if at least the nucleoprotein band (49 kD) could be visualized.

Results

Comparison of different cell fixation methods for IFA studies

The panel of 89 human sera was first tested in the IFA using either acetone (20°C) or ethanol (-70°C) fixed Vero E6 cells. The results obtained are summarized in Table 1. Of these sera, 54 were negative and 39 were positive in both the IFA systems. Three sera exhibiting low titers in the 'acetone IFA', were negative in the 'ethanol IFA' and also in Western blotting analysis. Only one of these could be confirmed to be positive in Western blotting analysis. The six sera showing 'atypical' reactivity in

the 'acetone IFA' were negative in the 'ethanol IFA' and also in Western blotting analysis. For further experiments the 'ethanol-IFA' was used and 'atypical' scores were considered negative.

Table 1. IFA results of sera of people 'at risk' or suspected of HV infection, tested on 76-118 virus-infected cell fixed with acetone (20°C) and ethanol (-70°C)

acetone (20°C)	positive	positive[a]	negative	'atypical'[b]	negative	negative
ethanol (-70°C)	positive	negative	positive	negative	'atypical'	negative
n = 102	39	3	0	6	0	54

[a] Acetone-IFA' titers: 16-32, 1 positive in Western blotting analysis.

[b] Atypical pattern of immunofluorescence, nagative in Western blotting analysis.

Comparison of IFA serum titers with serum titers in the respective ELISA systems

Comparison of IFA and cell-ELISA titers indicated that there was a good correlation between the results obtained in these assays (Fig. 1A). The IFA proved more sensitive, since all samples positive by the cell-ELISA were also positive in the IFA, but 11 samples with relatively low IFA titers (20-80) were not positive in the cell-ELISA. Nine of these were also tested by Western blotting analysis and shown to be positive (Table 2). Comparison of IFA and competition-ELISA titers showed a correlation between the two assays. However, the competition-ELISA proved less sensitive since it failed to detect 11 samples with low antibody titers (20-80) and two with high antibody titers (640) in the IFA (Fig. 1B) which also proved to be positive

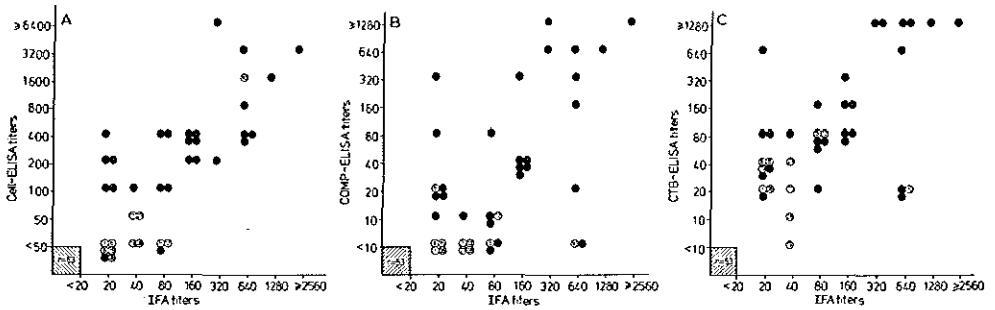

Figure 1. Relationships between antibody titers of sera from people at risk or suspected of HV infection by the IFA, cell-ELISA, competition-ELISA and CTB-ELISA. ○, Tested in Western blotting; ●, not tested in Western blotting. Numbers mentioned in the circles refer to Table 2.

in the Western blotting analysis (Table 2). Comparison of IFA and CTB-ELISA antibody titers indicated that there was a good correlation between the results of these two tests. The values found in the CTB-ELISA were generally higher than those found in the IFA (Fig. 1C). However, one sample with a titer of 40 in the IFA , which

was confirmed negative by Western blotting analysis, proved to be negative in the CTB-ELISA.

Comparison of titers in the respective ELISA systems

Comparison of the cell-ELISA and competition-ELISA antibody titers on the one hand and the CTB-ELISA on the other hand, showed that there was a correlation between the CTB-ELISA and the other two assays, but that the CTB-ELISA was the most sensitive. The cell-ELISA and the competition-ELISA failed to detect eleven and thirteen samples, respectively, which showed low antibody titers (20-80) in the CTB-ELISA (Fig. 2A, B). Nine and ten samples were confirmed positive by Western blotting analysis, but one sample, (13), was negative by Western blotting analysis (Table 2). Comparison of the cell-ELISA and the competition-ELISA titers indicated that these two assays yielded the same results (Fig. 2C). However, with five low titered sera and one serum with a high titer in the cell-ELISA, discrepant results were observed.

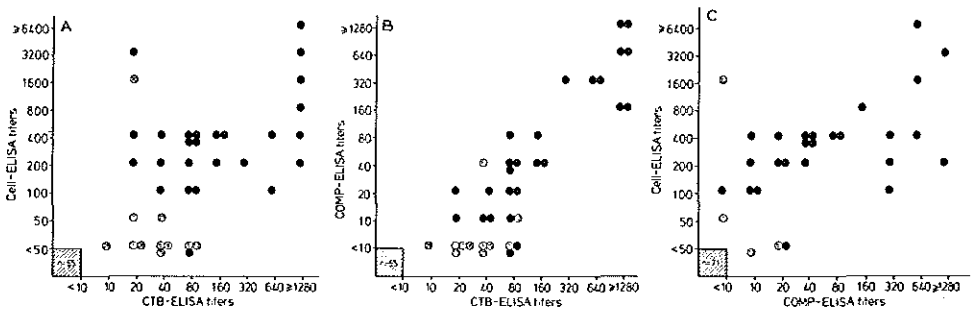

Figure 2. Relationships between antibody titers of sera from people at risk or suspected of HV infection by CTB-ELISA, cell-ELISA, competition-ELISA and between competition-ELISA and cell-ELISA. ○, Tested in Western blotting (Table 2); ●, not tested in Western blotting.

Discussion

In the present paper we have evaluated different IFA and ELISA systems for the demonstration of antibodies to HV in a selected panel of human sera, collected from people at risk or suspected of HV infection. Hantaan virus (strain 76-118) was selected for this study, since it has been shown that this virus exhibits a wide antigenic cross-reactivity with HV strains from other geographical areas [Schmaljohn et al., 1985; Lee et al., 1985]. First it was demonstrated that without serious loss of sensitivity ethanol (-70°C) fixation of HV-infected cells yielded fewer 'atypical' and non-specific reactions in IFA than the commonly used fixation with acetone (20°C). Therefore we speculate that the so-called 'atypical' IFA patterns observed were non-specific, although we cannot completely rule out the possibility that low-titered

Table 2. Western blotting analyses of sera from people 'at risk' or suspected of HV infection, with certain discrepancies between IFA and ELISA results.

Serum No.	Western blotting analysis	IFA[a] acetone (20°C)	IFA[b] ethanol (-70°C)	cell[b] ELISA (≥50)	competition[b] ELISA (≥10)	CTB ELISA (≥10)
1	+	128	20	-	-	10
5	+	64	20	-	-	80
6	+	128	20	-	40	40
7	+	16	40	50	-	20
8	+	128	20	-	-	40
10	+	256	640	1600	-	20
12	+	64	80	-	10	80
13	-	64	40	-	-	-
15	+	32	20	-	-	40
27	+	16	40	-	-	40
28	+	64	40	-	-	10
36	+	16	20	-	-	20

[a] Performed at ITM.
[b] Performed at RIVM.

antibodies to certain HV strains, which might give this aberrant pattern, were not recognized in the other assay systems. A comparison of the respective ELISA systems on the one hand, and of the ELISA's with the 'ethanol-IFA' on the other hand, showed that the three ELISA systems gave essentially the same results as the IFA but that only the CTB-ELISA was equally or more sensitive. No non-specific reactions were observed with this CTB-ELISA using the panel of human sera, as was confirmed by Western blotting analysis. Apart from the relatively high sensitivity and good correlation with the most commonly used test - the IFA - the clear advantages of the CTB-ELISA are the relative ease of performance and its standardization and automation. It was found that antibody-coated microtiter strips could be stored for over three months at -20°C without significant loss of antibody activity (data not shown). These strips may also be used for the detection of antigen in clinical specimens using the same test system with a known positive serum, although a confirmatory test would be needed if antibody preparations from other than specified pathogen free (SPF) animals were used. For this purpose a cocktail of polyclonal antibody preparations, preferably against a selection of geographical different virus strains, could be produced in SPF rats. An additional advantage of the CTB-ELISA is that, without the need of species-specific conjugates, sera from different species can be tested. We have confirmed this by testing positive rodent sera (data not shown), and it is of special importance for HV infections since these

viruses occur in a wide range of hosts [Van der Groen, 1986], and may occur as contaminants in biological products in, or with materials from, different animal species.

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Serum antibodies to structural proteins of hantavirus arise at different times after infection

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Journal of Medical Virology 1992, 37:283-287

Summary

An enzyme-linked immunosorbent assay (ELISA) was developed for the quantification of serum antibodies against group-specific epitopes of the glycoproteins (G1, G2) and nucleoprotein (NP) of the genus *Hantavirus*. This assay was used to study the kinetics of the development of serum antibodies after natural infection with Puumala-like virus in human. To this end a panel of 34 serum samples collected from individuals at different times after natural infection was tested in the ELISA. The samples were also tested for specific IgM and IgG levels against Puumala-like virus, which provided confirmatory data about the presumed timing of infection. It was shown that serum antibodies against the G1 epitope were present in the acute and early convalescent period just before antibodies to the NP epitope could be demonstrated. In contrast, antibodies to two G2 epitopes were present not earlier than in the convalescent and late convalescent period. Since all these categories of antibodies seem to persist for long periods, antibodies against the G1 epitope and the NP epitope may be of specific diagnostic value. Furthermore, levels of G1-specific antibodies and antibodies to either NP or G2 may allow estimation of the time elapsed following initial infection.

Introduction

Haemorrhagic fever with renal syndrome (HFRS) in Western Europe is predominantly caused by Puumala-like virus, of the genus *Hantavirus*, which belongs to the family *Bunyaviridae* [Groen et al., 1991a; Zoller et al., 1989; Niklasson et al., 1990]. At least five different serotypes have been proposed for the genus *Hantavirus*: Hantaan-like virus, Seoul-like virus, Puumala-like virus, Prospect Hill virus and Leaky virus [Lee et al., 1990; Sugiyama et al., 1986]. So far only Hantaan-like virus, Seoul-like virus and Puumala-like virus have been associated with clinical manifestations in humans [Clement and Van der Groen, 1987; Oster-

haus et al., 1989; Groen et al., 1991b; Niklasson and LeDuc, 1987; Tsai, 1987].

Several serological techniques have been described for the detection of specific antibodies to Hantavirus, including immunofluorescence assays (IFA), plaque reduction neutralization assays, enzyme-linked immunosorbent assays (ELISA), Western blot assays and haemagglutination inhibition tests [Lee et al., 1985; Goldgaber et al., 1985; Dantes et al., 1987; Takahashi et al., 1986; Groen et al., 1989; Zoller et al., 1989]. None of these assays have allowed the specific identification of antibodies against the individual proteins of the virus. In other viral systems like rabies and measles it has been shown that antibodies directed to different structural proteins, arise at different times after vaccination [Grassi et al., 1989; De Vries et al., 1990]. In case of human immunodeficiency virus (HIV) infection in humans, the ratio of antibody levels to different structural proteins of the virus may have significant diagnostic and prognostic values [Portera et al., 1990; Teeuwsen et al, 1991]. Since IgM antibodies may persist for long periods after Hantaan virus (HV) infection [Niklasson et al., 1990; Ivanov et al., 1988], their diagnostic value for identification of acute HV infection is of limited value. We were, interested to evaluate whether measurement of antibodies directed to different structural proteins of HV may allow the estimation of the interval elapsed after initial HV infection.

We here describe the evaluation of an ELISA system, based on monoclonal antibodies (MCA) directed to group-specific determinants on the G1, G2 and NP of Hantaan virus, for the quantification of antibodies specific for these respective structural proteins of this virus. With this ELISA the kinetics of antibody development to these proteins after natural infection in humans were followed. The results were compared with those obtained with the same serum samples in the recently described ELISA for the detection of HV-specific IgM and IgG antibodies [Niklasson et al., 1990]. This comparison indicates that the ELISA system can define the stage of HV infection more accurately than the IgG/IgM detection method.

Materials and methods

Antigen

Viral antigen was prepared from HV Puumala-like virus (strain Hällnäs) infected Vero E6 cells. When 80% of the cells were infected, as determined by IFA, they were solubilized with a mixture of detergents as described previously, and after low speed centrifugation the supernatant was used as antigen in the assay systems [Groen et al., 1989].

Antisera

Specific rabbit immune serum against Puumala virus was prepared as described

[Groen et al., 1991b]. MCA reactive with group-specific epitopes of the structural proteins of Hantaan virus, were generated as previously described [Arkawa et al., 1989; Ruo et al., 1991]. For the inhibition ELISA described below, four MCA were selected against: G1 (8B6), G2d (3D7), G2f (8E10) and NP (GB04). All these MCA were IgG1 isotypes and their specificity for the respective proteins had been demonstrated by immune precipitation. The difference among the two G2 epitopes have been evaluated in a competitive ELISA [Arikawa et al., 1989].

Serum samples

Thirty-four serum samples, positive by IFA to Puumala virus were collected from 23 HFRS patient in the acute (0-2 weeks), early convalescent (3-5 weeks), convalescent (6-52 weeks) and late convalescent (>52 weeks) phase of the disease. From 13 patients one sample was obtained, from nine patients two samples and from one patient three samples. All patients had developed symptoms indicative for HFRS in The Netherlands or Belgium in the preceeding years [Groen et al., 1991a; Groen et al., 1991b; Osterhaus et al., 1989; Jordans et al., 1991a; Jordans et al., 1991b] and had probably been infected with a Puumala-like virus due to contact with wild rodents [Groen et al., 1991a].

IgM capturing ELISA

An IgM capturing ELISA was carried out with minor modifications essentially as described earlier [Niklasson et al., 1990]. Briefly, goat anti-human IgM (μ-chain-specific; Cappel Cooper Biomedical, West-Chester, PA, USA) was coated onto microtiter plates for 18 hrs at 4°C in 0.1 M carbonate/bicarbonate buffer pH 9.6 with 50 μl volumes/well. After washing the plates with tap water containing 0.05% Tween 80, 100 μl volumes of ELISA-buffer (PBS supplemented with 0.1% Triton, 0.1% Tween 20, 5% NaCl, 1% goat serum and 10% Fetal Calf Serum [FCS]) (EBT) was added per well, incubated for 1 hr at 37°C and washed three times. Fifty μl volumes of 1:10 diluted human serum in EBT in duplicate was added, incubated for 4 hrs at 37°C, washed three times and 50 μl volumes of 1:10 diluted Puumala virus in EBT plus 1% normal human serum (NHS) and control antigen was added to each well of the serum sample and incubated for 18 hrs at 4°C. After washing, the binding of the antigen was detected by adding 50 μl of 1:100 diluted rabbit anti-Puumala antibodies, incubated for 1 hr at 37°C, followed by washing and incubation with 50 μl 1:300 diluted swine anti-rabbit HRPO (Dakopatts, Glostrup, Denmark) for 1 hr at 37°C. Plates were developed with TMB as substrate [Groen et al., 1989]. IgM values of each serum were expressed as the levels of the optical density on positive antigen divided by the optical density on control antigen.

Indirect IgG ELISA

The indirect ELISA for the detection of specific IgG antibodies was carried out in a microtiter system essentially as the indirect ELISA for the detection of total Ig described previously [Groen et al., 1991b]. Biotin labeled sheep anti-human IgG was used as a conjugate instead of sheep anti-human Ig HRPO. Levels of IgG antibodies were calculated as described above.

Inhibition ELISA

Inhibition ELISA in which epitope-specific antibodies could be detected was carried out in a microtiter system. Briefly, plates (Costar, Cambridge, MA, USA) were coated with 50 µl detergent solubilized Puumala virus 1:100 dilution in 0.1 M acetic buffer pH 5.5 for 18 hrs at 4°C. Antigen was removed and 200 µl/well fixative was added, ethanol was used for MCA 8B6 and 4% paraformaldehyde for the MCA 3D7, 8E10 and GB04. After 10 min at room temperature the plates were washed with demineralized water containing 0.05% Tween 20 (Merck, Schuchardt, Germany) (DWT), blocked with 100 µl/well PBS 0.1% Tween 20, 0.1% Triton X-100 and 10% FCS (PBS-T) and incubated for 1 hr at 37°C. The plates were washed three times with DWT and then incubated with 50 µl human serum prediluted 1:10 in fourfold with PBS-T. The plates were then incubated at 37°C, after 4 hours 25 µl of the serum was removed, 25 µl of the optimal working dilution of ammonium sulphate purified MCA was added to each well (8B6, 3D7, 8E10 and GB07) and incubated overnight at 4°C. Plates were washed three times with DWT and 50 µl volumes of HRPO conjugated rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark) were added for 1 hr at 37°C. Plates were developed after washing with DWT, using the TMB substrate and read at A450 nm as described [Groen et al., 1989]. Reduction percentage was calculated on the basis of the formula:

$$\% \text{ inhibition} = \frac{\text{OD450 negative control serum - OD450 test serum}}{\text{OD450 negative control serum}} \times 100\ \%$$

Results

Development of HV-specific IgM and IgG serum levels

A collection of 34 serum samples which had been shown positive in the indirect ELISA and IFA for the detection of HV-specific antibodies, was divided into four groups according to the presumed period that had elapsed between the onset of clinical symptoms and the collection of the samples: acute, early convalescent, convalescent and late convalescent (for criteria see "Materials and Methods").

HV-specific IgM and IgG levels were quantified in all the 34 samples. It was shown that the IgM levels in the acute phase were relatively high (mean levels 5.6), and decreased gradually to disappear finally in the late convalescent phase (mean levels 1.1) (Fig. 1). The HV-specific IgG levels were relatively low in the acute phase (mean levels 2.1) and increased gradually to reach their maximum values in the late convalescent phase (mean levels >11). These data indicate that as in several other virus infections, the quantitation of levels of HV-specific IgM and IgG may give an indication about the stage of HV infection.

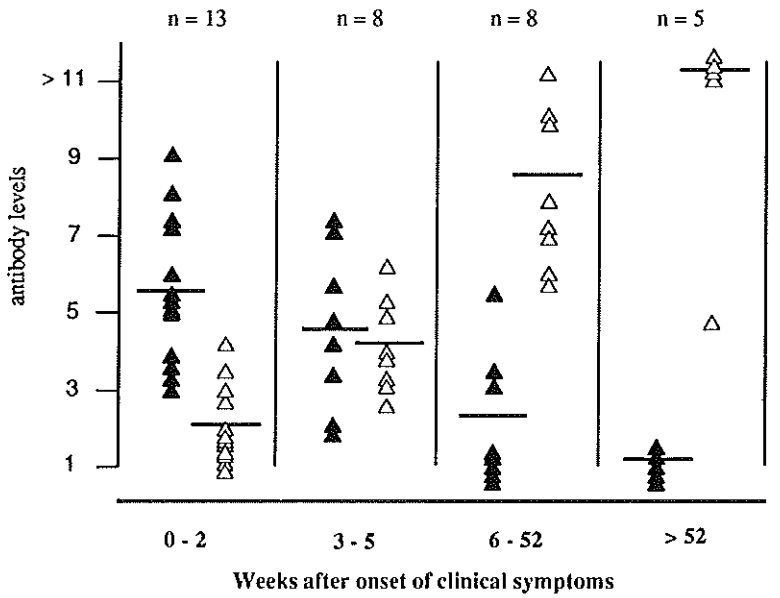

Figure 1. Levels of HV-specific IgM (▲) and IgG (△) antibody levels in sera from individuals infected with Puumala-like virus, in the acute (0-2 weeks), early convalescent (3-5 weeks), convalescent (6-52 weeks) and the late convalescent (>52 weeks) phases.

Development of serum antibodies to individual HV structural proteins

The 34 serum samples divided into the four groups, were tested in the different inhibition ELISA for the detection of antibodies directed against G1, G2 and NP proteins. The reactivities of these serum samples in the respective assays are shown in Figure 2. Within two weeks after presumed infection, antibodies against the conserved epitope on G1 were detectable in all the serum samples tested (mean inhibition level 58%). Mean inhibition levels against this epitope gradually increased in the convalescent and late convalescent phase (mean inhibition levels >80%). Antibodies to two different epitopes of the G2 (G2d and G2f) developed more slowly after infection: in the acute and early convalescent phase no or very low antibody levels were found (mean inhibition levels <10 %). In the convalescent phase, they gradually increased (mean inhibition levels 20-40%) to reach the highest levels in the

late convalescent phase (mean inhibition levels 30-60%). Antibodies against the epitope on the NP were detectable in all the sera in the acute phase (mean inhibition level 34%) and gradually increased in the subsequent stages to reach maximal values in the late convalescent phase (mean inhibition level 76%).

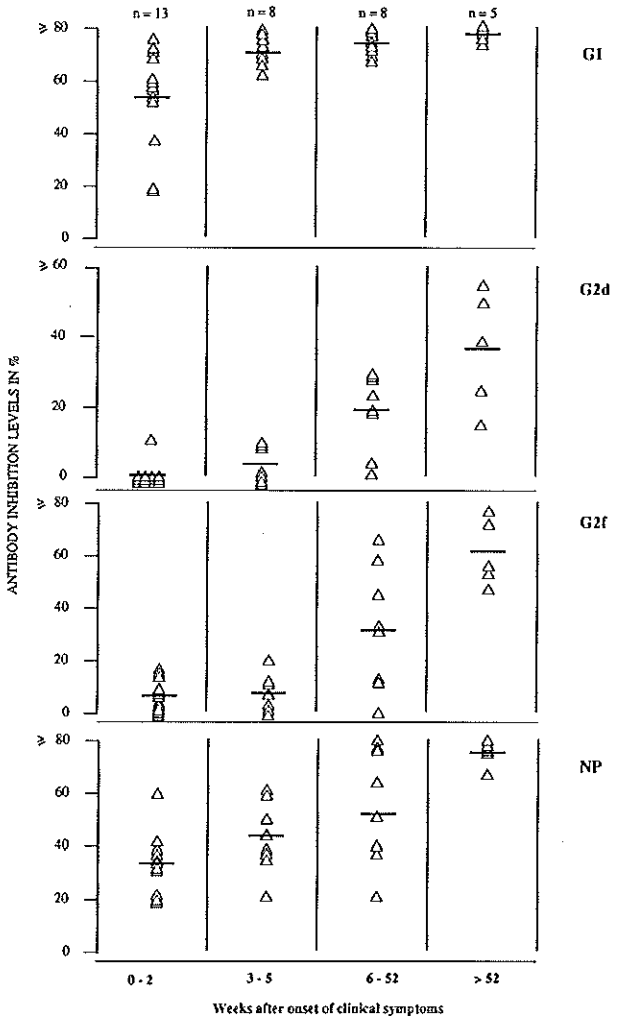

Figure 2. Antibody levels (△) in sera from individuals infected with Puumala-like virus to group-specific epitopes on the G1, G2d, G2f and NP, measured in the inhibition ELISA system and expressed as inhibition percentages.

Discussion

We analysed total IgM and IgG serum levels to HV and antibody levels to

conserved epitopes of different structural proteins of HV in 34 sera from HFRS patients at different stages of Puumala-like infection. It was shown that, as in many other virus infections, the highest levels of specific IgM was detectable in the acute phase of HV infection when HV-specific IgG antibodies were virtually absent. With the disappearence of HV-specific IgM antibodies in the period after acute infection, HV-specific IgG antibodies increased. Therefore, as in previous studies by others, we confirmed the presumed period that had elapsed after the onset of the initial clinical symptoms of HFRS using these assays [Niklasson et al., 1990; Ivanov et al., 1988].

Evaluation of the potential of the newly developed inhibition ELISA, which detects antibodies to conserved epitopes of the different structural proteins of HV, indicated that with this system different levels of antibodies to these proteins could be measured at different stages of the infection: Antibodies against the G1 and the NP epitopes could already be detected in the acute phase. In contrast, antibodies to both the G2 epitopes were virtually undetectable before the convalescent period. This implies that the detection of G1- and NP-specific antibodies in this ELISA is of major value in the diagnosis of HV infection. The distribution of the antibodies to the four different epitopes of the respective proteins, permits the use of such data for the estimation of the period elapsed after initial infection. The acute and early convalescent stages of HV infection in particular can be identified more easily by this ELISA than with classical HV-specific IgM/IgG detection. As shown in Figures 1 and 2, this may have diagnostic and prognostic implications. It may also have consequences for therapeutic approaches to be chosen, which also depend on the stage of infection. Since the MCA used in the ELISA system all detected group-specific epitopes present on five different serotypes of hantavirus, it may be expected that the system may also be used for the diagnosis of other HV infections than those involving the Puumala-like virus. At present it is not clear why antibodies against the respective epitopes on the different proteins cannot be measured to the same extent at different stages of the infection. An analogous situation has been described for the development of antibodies specific for the different HIV proteins. The mechanism underlying this phenomenon may, however, be different from that observed in HV infection. In HIV infection, persistence is a major feature involved in pathogenesis, which does not seem to be the case in human HV infections. In the case of HV infection, the differences found in antibody levels against the epitopes on different structural proteins may reflect the later appearence of G2-specific antibodies during infection due to a delay in their production, different sensitivities of the respective test systems or the complexing of circulating antibodies by G2 antigen early in HV infection. However, the assay system in its present form is useful for diagnostic purposes. To investigate the system, a more extensive serum panel, also including samples from humans and rodents infected with Puumala-like virus or other serotypes of HV, should be tested to finally assess its potential. This ELISA may

also prove to be useful for the evaluation of antibody responses to different HV proteins involved in eliciting protection after vaccination with candidate vaccines.

Aknowledgments

We thank Mr. A. Lefevré and Mr. R. Stokking for their technical assistance, and Ms. C. Kruyssen and Ms. M.C. Eskens for help in preparing the manuscript.

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Class and subclass distribution of hantavirus-specific serum antibodies at different times after the onset of nephropathia epidemica

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Journal of Medical Virology 1994, 43:39-43

Summary

Sera from Dutch and Belgium individuals who had suffered from nephropathia epidemica (NE), a mild form of haemorrhagic fever with renal syndrome (HFRS), were tested for the distribution of classes and subclasses of hantavirus (HV) specific antibodies at different times after the onset of the disease, with class- and subclass-specific Ig capture enzyme-linked immunosorbent assays (ELISAs). In the acute, early convalescent and convalescent phases, predominantly specific IgA, IgM and IgG3 antibodies were detected. Specific IgG2 antibodies were only detected at low levels in the early convalescent and convalescent phases. In the late convalescent phase specific IgG1 and IgG3 antibodies were found, whereas in the late post convalescent phase only specific IgG1 antibodies proved to be present. Specific IgG4 antibodies were not detected in any of the respective phases. These data show that the simultaneous determination of classes and subclasses of HV specific serum antibodies allows the estimation of the time elapsed after the onset of NE.

Introduction

The mild form of haemorrhagic fever with renal syndrome (HFRS) found in humans in Western Europe and known as nephropathia epidemica (NE), is caused by viruses similar or identical to Puumala virus, which belongs to genus *Hantavirus* (HV) of the *Bunyaviridae* family [Clement and Van der Groen, 1987; Tsai, 1987; Zoller et al., 1989; Niklasson et al., 1990; Groen et al., 1991a,b]. HV infections can be diagnosed by detection of specific IgM and rising IgG serum antibodies [Niklasson et al., 1990; Groen et al., 1992], HV isolation and by the recently described polymerase chain reaction (PCR) [Grankvist et al., 1992]. Recently we have showed that serum antibodies to different structural proteins of HV, as measured by a competitive ELISA, arise at different times after infection. This allows a more accurate determination of the time that elapsed after infection than

traditional measurement of specific IgG and IgM antibodies [Groen et al., 1992]. This proved to be important since it has been shown that IgM antibodies may, in rare cases persist for long periods after infection and paired serum samples, especially in the acute phase, are often not available [Ivanov et al., 1988; Niklasson et al., 1990]. Virus-specific IgA serum antibodies may be a reliable indicator for recent virus replication at mucosal surfaces in many systems [Sarnesto et al., 1985a,b; Friedman et al., 1989; Coulson et al., 1990; Pozetto et al., 1990], but have so far not been implicated in the serological diagnosis of HV infections. An IgG avidity assay proved to be a useful tool for assessing the time after rubella virus infection [Devey et al., 1988; Thomas et al., 1992]. The distribution of the four subclasses of specific IgG has been shown to change during and after several virus infections and their measurement may therefor contribute to the estimation of the time elapsed after initial virus infection [Sundqvist et al., 1984; Sarnesto et al., 1985a; Linde et al., 1987; Sällberg et al., 1990].

We examined the distribution of Ig classes and IgG subclasses of antibodies in 35 human sera collected from 18 Dutch and Belgium individuals [Clement and Van der Groen, 1987; Osterhaus et al., 1989; Groen et al., 1991a,b; Jordans et al., 1991], in the acute and convalescent phases of NE, using class- and subclass-specific Ig capture ELISAs. It is shown that the simultaneous determination of classes and subclasses of serum antibodies, allows an accurate estimation of the time that has elapsed after the onset of NE.

Materials and methods

Antigen

Vero E6 cells infected with Puumala-like virus (strain Hällnäs) was used as source of viral antigen. When 80% of the cells were infected, as determined by immunofluorescent assay (IFA), they were solubilized with a mixture of detergents, and after low speed centrifugation the supernatant was used as antigen in the assay systems described previously [Groen et al., 1989].

Serum specimens

The 35 human serum specimens used in this study were collected from 18 individuals who had suffered from a disease with a differential diagnosis of leptospirosis, acute nephritis or acute renal insufficiency. All individuals were residents of The Netherlands and Belgium and their sera scored positive in the IFA [Clement and Van der Groen, 1987; Osterhaus et al., 1989; Groen et al., 1991a,b; Jordans et al., 1991]. The epidemiological, clinical and serological data of these cases are summarized in Table 1. The sera were divided into five groups according to the

time that had elapsed after onset of clinical symptoms of NE. Eight samples were obtained during the acute phase (1-10 days after the onset of disease), nine during the early convalescent phase (11-25 days), seven during the convalescent phase (26-65 days), five from the late convalescent phase (10-35 weeks) and six from the late post convalescent phase (>52 weeks).

Table 1. Epidemiological, clinical, and serological data of the NE patients from The Netherlands and Belgium studied in this evaluation.

Sample	Individuals		Clinical			Serum antibody titer to (IFA)	
	Sex	age (years)	Duration[1] (days)	Peak Creatinine (µmol/l)	Peak Proteinuria (gr/l)	HTN[3] (strain 76-118)	PUU[3] (strain Hällnas)
02d	female	31	21	1104	15.0	60	180
03l	female	35	21	293	8.9	1620	>4860
27r	female	46	14	395	nd[4]	180	1620
36w	female	50	18	757	0.4[2]	60	1620
21m	female	63	12	355	trace	1620	>4860
35o	male	17	15	217	1.8[2]	180	1620
18k	male	21	15	412	trace	180	14580
38k	male	22	13	257	10.0	<20	540
16j	male	27	23	257	2.8[2]	540	4860
23c	male	29	9	266	+++	512	2048
05g	male	30	17	378	13.8	540	540
32m	male	32	8	126	1.0[2]	128	4096
01s	male	34	30	986	10.0	540	540
17r	male	40	50	775	30.4	32	256
15d	male	46	15	451	+++	180	4860
22b	male	46	16	566	nd	32	>4860
37r	male	55	19	646	9.4	60	>540
25r	male	61	31	1300	1.0[2]	60	>1620
n=18	72% (male)	38 (mean)	30 (mean)	94% (>133 µmol/l)		89% (highest titer against PUU)	

[1]from the onset of clinical symptoms until normalization
[2]gr/24hr
[3]HTN = Hantaanvirus; PUU = Puumalavirus (first serum sample tested)
[4]nd= not done

Capturing ELISAs for measuring Ig classes and subclasses in serum

The ELISA for the measurement of specific IgM serum antibodies was carried out described previously [Groen et al., 1992]. For the measurement of specific IgA, goat anti-human IgA (Cappel Cooper Biomedical, West-Chester, PA, USA) was used as capture antibody instead of goat anti-human IgM.

ELISAs for the determination of IgG subclasses, werecarried out as follows: Avidin (Sigma, A9390, St.Louis, USA) was coated onto microtiter plates for 1 hr at 37°C in PBS pH 7.2 using 50 µl (20 µg/ml) volumes/well. After washing the plates with tap water containing 0.05% Tween 80 (WS), 50 µl biotin labelled goat anti-mouse IgG1 or IgG2a was added, after dilution 1:100 in ELISA-buffer (PBS supplemented with 0.1% Triton, 0.1% Tween 20, 1% fetal calf serum, 0.1% milk powder and 5% normal goat serum) (EBT). The plates were incubated for 4 hrs at 37°C and washed three times with WS. Fifty µl volumes of monoclonal antibodies (Moab) to human IgG subclasses (1 to 4) diluted 1:100 in EBT were added and the plates were incubated again for 1 hr 37°C. The specificity of the Moab's purchased

from Seralab (Sussex, UK) were as follows: anti-human IgG1 (hybridoma JDC-1; IgG1), IgG2 (hybridoma 31-7-4; IgG1), IgG3 (hybridoma C3-8-80; IgG2a) and IgG4 (hybridoma 1D7; IgG1). The plates were subsequently washed and incubated overnight at 4°C with 1:100 dilution of the human serum in EBT. The measurement of subclass antibodies was carried out as described for the measurement of specific IgM antibodies [Groen et al., 1992] using a multiscanspectrophotometer. The values of IgA, IgM and the four subclasses of IgG were expressed as the optical density measured at 450 nm (OD 450 nm) on positive antigen subtracted with the OD 450 nm of the control antigen.

Results

IgA serum antibodies

Specific IgA antibodies were detected in all the sera collected during acute clinical symptoms of HV infection and in the early convalescent phase, with geometric mean OD 450 nm values (GMV) of 1.079 and 1.022 respectively. IgA antibodies were also detected in the majority of the sera collected in the convalescent phase, with a GMV of 0.772. These antibodies were not be detected in the late and late post convalescent phases (Fig. 1).

IgM serum antibodies

The distribution of specific IgM antibodies largely paralelled that of the specific IgA antibodies in the sera tested: all had specific IgM serum antibodies in the acute, early convalescent and convalescent phases (GMV 1.253, 1.076 and 0.990 respectively), which were absent in the late convalescent phase and detectable in only two of the sera collected in the late post convalescent phase.

I*gG serum antibodies and their subclass distribution*

Specific IgG1 antibodies were detected in almost all the sera tested. Three out of seven of the sera collected during the acute phase contained specific IgG1 antibodies (GMV 0.300). Also in most of the sera collected in the early convalescent phase, IgG1 antibodies were detected (GMV 0.366), but all the sera collected in the convalescent, late convalescent and late post convalescent phases contained specific IgG1 antibodies with an increase of average OD 450 nm values towards the later phases (GMV 0.481, 0.656, 0.923 respectively) (Fig. 1). Low levels of specific IgG2 serum antibodies were detected in a minority of the sera collected during the early convalescent and convalescent phases (GMV 0.173 and 0.196 respectively). Virtually no specific IgG2 serum antibodies were found during the two later phases (GMV 0.054 and 0.072 respectively) (Fig. 1). Specific IgG3 antibodies were detected in all or most of the sera collected in the acute, early, convalescent and late

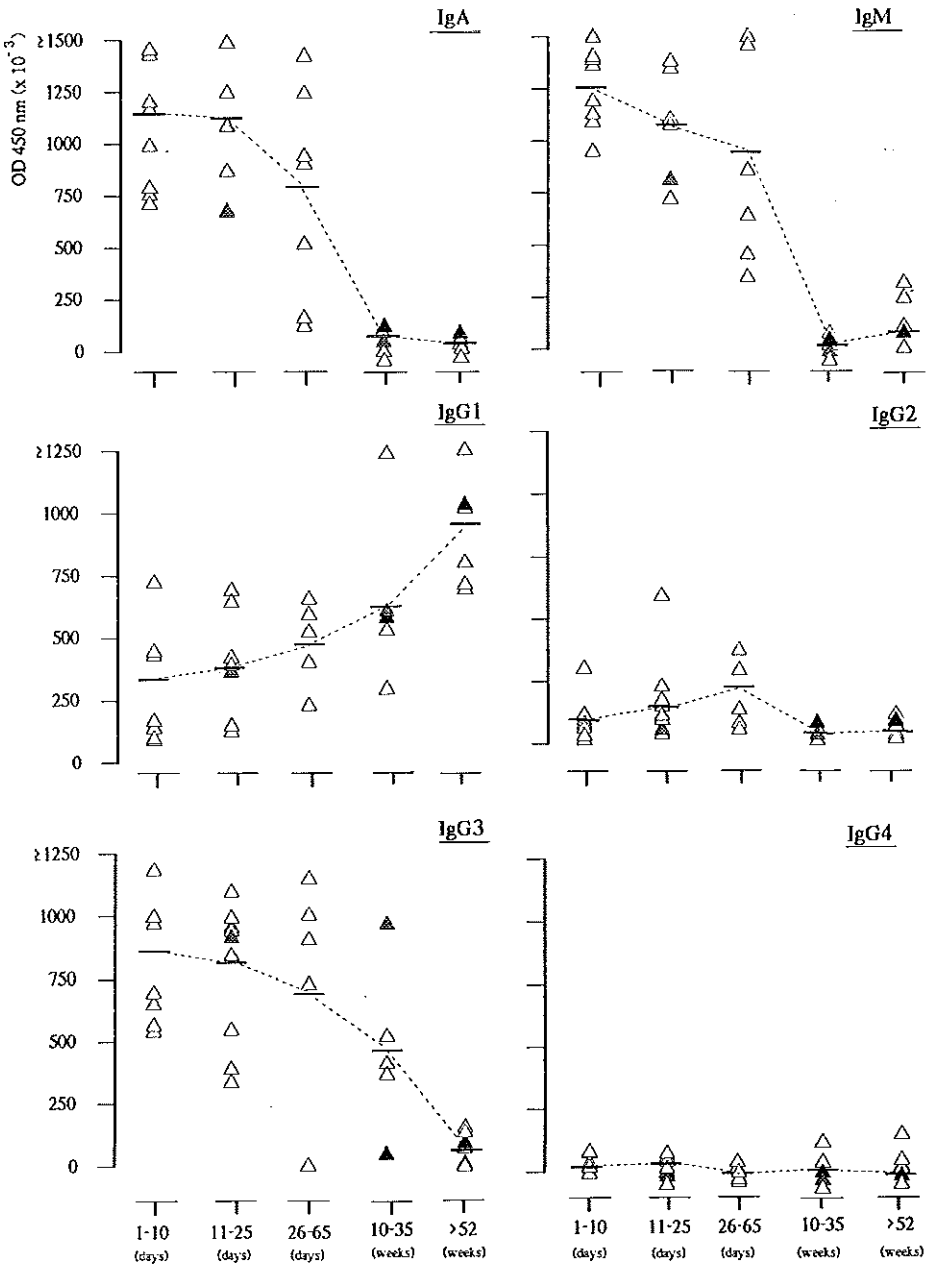

Figure 1. Levels of HV specific (△) class and subclass antibodies in serum samples collected at different times after the onset of NE. Results of assays carried out with the sera collected from the same individual at different times, are indicated for three individuals (▲▲▲).

convalescent phases (GMV 0.804, 0.786, 0.703 and 0.471 respectively). They were virtually absent in the sera collected in the late post convalescent phase (Fig. 1). Specific HV IgG4 serum antibodies were not be detected in any of the samples collected during the respective phases (Fig. 1).

Discussion

Simultaneous measurement of Ig classes and IgG subclasses of serum antibodies allows an estimation of the time that has elapsed after the onset of NE. Although several studies have shown that the measurement of IgM serum antibodies, may give an indication about the acuteness of HV infection [Ivanov et al., 1988; Niklasson et al., 1990; Groen et al., 1992], we have recently shown that the measurement of serum antibodies to different structural proteins of HV may provide a better tool in the determination of the time that had elapsed after the initial HV infection [Groen et al., 1992]. Like the measurement of specific IgM serum antibodies, the measurement of HV-specific IgA serum antibodies, proved to be an adequate method for determining the stage of HV infection since HV-specific IgA serum antibodies were detected in all the sera collected in the acute and early convalescent phases, but not in those collected in the late convalescent and late post convalescent phases (Fig. 1). A similar observation was made for other virus infections which primarily infect mucosal surfaces like infection with measles-, rota- and enteroviruses [Friedman et al., 1989; Coulson et al., 1990; Pozzetto et al., 1990].

The value of measuring specific IgG subclasses in combination with the measurement of specific IgM and IgA antibodies, to determine the stage of HV infection, was studied. IgG1 is the major subclass of specific antibodies found in response to acute viral infections [Linde et al., 1987]. Carbohydrate antigens mainly elicit IgG2 antibodies [Asano et al., 1988]. Of all subclasses IgG3 is of special interest due to its more efficient binding to monocytes, its relatively high neutralizing capacity [Mathiessen et al., 1988], its association with clinical recovery and its value for early diagnosis [Asano et al., 1987; Linde et al., 1987; Arico et al., 1991]. The distribution of the four IgG subclasses of serum antibodies over time was similar to that observed in a number of other non-persistent virus infections [Julkunen et al., 1985; Sarnesto et al., 1985a,b; Thomas and Morgan-Capner, 1988]. Specific IgG1 and IgG3 serum antibodies were shown to display opposite kinetics: specific IgG1 antibodies arose slowly to reach high levels long after infection, whereas specific IgG3 antibodies were predominantly found soon after infection to disappear completely in later stages.

The determination of the ratio between IgG3 and IgG1 may help in the discrimination between early stage and persistent IgM. This was e.g., shown in two

individuals with persistent low levels of IgM in the late post convalescent phase, who both showed a IgG3/IgG1 ratio of 0.1, whereas the mean ratio's of the acute and early convalescent phases were 3.9 ± 2.1 and 2.7 ± 1.6, respectively.

It was also demonstrated that specific IgG2 and IgG4 serum antibodies are less pronounced in the antibody response to HV infection. Viral-specific IgG4 antibodies have been demonstrated in infections with viruses which tend to persist in the host-like Hepatitis B, Herpes simplex and Varicella-zoster viruses [Asano et al., 1987; McBride and Ward, 1987; Asano et al., 1988; Sällberg et al., 1990]. Our data suggest that the distribution of serum antibodies to HV in humans after natural infection largely parallels the antibody responses found after non-persistent virus infections of mucosal surfaces and therefor it may be speculated that also HV infections do not persist after recovery from clinical symptoms. It would also be interesting to compare the HV-specific subclass responses with an IgG avidity assay as described for rubella virus [Thomas et al., 1992]. This would be of special interest since it may be speculated that different serotypes of HV may be involved in HV disease in The Netherlands [Groen et al., 1991a] and that the infecting virus strain might influence the outcome of the IgG avidity assay.

Taken together it is concluded that the simultaneous measurement of Ig classes and IgG subclasses of serum antibodies, like the simultaneous determination of serum antibodies to structural proteins of HV, allows an accurate estimation of the time elapsed after onset of clinical symptoms of NE. This is important for the diagnosis and management of acute renal disease due to NE, since the discrimination between HV infection and other causes of acute nephritis on the one hand, and the determination of the stage of the disease on the other hand have direct consequences for therapy and prognosis.

Acknowledgments

The authors wish to thank Ms. C. Kruyssen for the preparation of the manuscript and Mr. A. Lefévre for technical assistance. This work was supported by a grant from the Dutch Veterinary Inspectorate.

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Histopathological findings and hantavirus antigen detection in kidney biopsies from patients with nephropathia epidemica

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Clinical Nephrology, 1995 submitted

Summary

Renal biopsies collected from thirteen patients with serologically confirmed Puumala virus (PUUV) infection in the acute stage and from ten PUUV seronegative patients with renal dysfunction who lived in the same PUUV endemic area in The Netherlands, were studied histologically, immunohistochemically and subsequently examined for the presence of viral antigen by immunoperoxidase staining. Interstitial haemorrhages and deposition of IgM, complement component C3 and fibrin in the tubular basement membrane, were only found in PUUV seropositive patients. In six of these patients viral antigen was detected in the cytoplasm of renal tubular epithelial cells with focal distribution in the cortical and medullary areas of the kidneys. Surprisingly, also in two of the ten PUUV seronegative patients, PUUV antigen was demonstrated, with essentially the same localization. Comparison of severity and extent of the lesions showed no significant differences between tubular, interstitial and glomerular abnormalities between both groups. These findings indicate that the demonstration of certain histopathological alterations and the detection of viral antigen by immunoperoxidase techniques in kidney biopsies, may be useful as additional tools for the diagnosis of PUUV infection.

Introduction

Nephropathia epidemica (NE), caused by Puumala-like viruses (PUUV), is considered to be the most frequently reported rodent-borne virus infection in Europe [Van Ypersele De Strihou and Mery 1989, Childs and Rollin, 1994]. PUUV are antigenically and genetically related to the other members of the genus Hantavirus, including Hantaan-like viruses and Seoul-like viruses which both cause haemorrhagic fever with renal syndrome (HFRS) [Lee et al., 1985; Puthavathana et al., 1992, 1993] and to Muerto canyon virus. The latter causes hantavirus pulmonary syndrome [Nichol et al., 1993]. Rodents are the major reservoir host species from

which hantaviruses are transmitted via aerosols to humans [Tsai, 1987]. In the rodent species hantaviruses usually cause subclinical and persistent infections. The spectrum of clinical symptoms caused in humans, may vary from subclinical or mild to severe. This depends largely on the viral subtype involved [Childs and Rollin, 1994]. The clinical diagnosis of NE may be confirmed by the demonstrating the development of PUUV specific serum IgG [Hedman et al., 1991].

In The Netherlands we identified more than 40 cases of NE in the last decade [Groen et al., 1995a]. Although the diagnosis of NE is usually not primarily made on the basis of histopathology [Lähdevirta et al., 1971; Collan et al. 1991], knowledge of HFRS related histopathology may have additional diagnostic value. In a recently established macaque model [Groen et al., 1995b] we showed that the development of histopathological abnormalities in the renal tubular epithelium, the presence of hantavirus antigen and RNA in the kidney, as well as the development of specific serum antibodies, clearly parallelled the findings in humans with NE [Groen et al., 1995b]. Here we describe histopathological and immunohistochemical changes as well as the detection of viral antigen in renal biopsies from patients with serologically confirmed and unconfirmed NE.

Materials and methods

Patients specimens

Two groups of patients were selected. The first consisted of 13 serologically confirmed PUUV (presence of PUUV specific IgG antibodies) infected patients from whom kidney biopsies had been taken in the acute stage of renal failure between three and eleven days after the onset of disease. Ten serum samples were obtained during this period and four serum samples were collected more than six months after the disease onset. The second group consisted of ten PUUV seronegative patients matched for age and sex and suffering from chronic renal failure or nephrotic syndrome. Both groups resided in a PUUV endemic area in The Netherlands [Groen et al., 1995a]. Patients with extensive glomerular abnormalities were excluded (Table 1). From all the patients of the second group kidney biopsies had also been taken when they exhibited acute disease symptoms. The biopsied kidney tissues and serum samples from the 23 patients, who all had been admitted to hospitals in PUUV endemic areas in The Netherlands had been collected for diagnostic purposes between 1977 and 1993. Histopathological findings in these biopsies from both groups, scored by routine nephropathological examination in the hospitals are summarised in Table 1.

Table 1 Morphological changes in kidney biopsies scored by routine histological analysis in the hospital, epidemiological data and PUUV specific serology of patients with NE and chronic renal failure

Group	Biopsy number	Histopathological diagnosis by light microscopic evaluation carried out in diagnostic hospital setting	Sex	Age	PUUV specific IFA (strain Hällnäs)		
				(years)	IgM	IgA	IgG
Positive	1	no abnormalities	male	34	-[R]	<20[R]	540
	2	no abnormalities	male	28	+	60	1620
	3	no abnormalities	male	48	+	180	540
	4	no abnormalities	female	46	+	60	180
	5	no abnormalities	male	36	+	540	540
	6	slight tubular interstitial nephritis + eosinophilic leucocytes	female	50	-[R]	<20[R]	≥1620
	7	slight tubular interstitial nephritis	male	32	+	≥20	180
	8	acute tubulointerstitial nephritis	male	21	+	60	≥1620
	9	acute tubulointerstitial nephritis	female	31	-[R]	<20[R]	180
	10	acute tubulointerstitial nephritis + eosinophilic leucocytes	female	54	+	540	540
	11	acute tubulointerstitial nephritis	male	30	-[R]	<20[R]	540
	12	tubular necrosis	female	43	+	540	≥1620
	13	interstitial nephritis, renal arteriosclerosis	male	61	+	180	≥1620
			(male) 62%	mean 40 ± 12			
Negative	14	minimal lesions	male	41	-	<20	<20
	15	acute tubulointerstitial nephritis	female	62	-	<20	<20
	16	granulomatous interstitial nephritis + eosinophilic leucocytes	female	67	-	<20	<20
	17	focal sclerosis	female	26	-	<20	<20
	18	focal segmental sclerosis	male	45	-	<20	<20
	19	focal segmental sclerosis	male	17	-	<20	<20
	20	focal segmental glomerular sclerosis	male	30	-	<20	<20
	21	ST I membranous glomerulopathy	male	74	-	<20	<20
	22	IgA nephropathy	male	20	NT	<20	<20
	23	IgA nephropathy	male	47	-	<20	<20
			(male) 70%	mean 44 ± 12			

[R]: retrospective serology
NT: not tested

Serology

Hantavirus specific IgA, IgG and IgM serum antibodies were measured by an indirect immunofluorescence and by μ-capturing ELISA respectively, as previously described [Groen et al., 1994, 1995b].

Histopathology and histochemistry

For histopathological analysis, kidney needle biopsy specimens had been collected, fixed in 10% buffered formalin and embedded in paraffin. The biopsies were cut into 4 to 5 μm sections and subsequently stained with haematoxylin-eosin and PAS before microscopic examination. Morphological lesions in coded samples were scored according to previously described histological characteristics [Collan et al., 1991] and according to an arbitrary scale from 0 to 3.

The direct immunofluorescence assays for the qualitative detection of total IgA, IgG, IgM, complement component C3 and fibrinogen were performed with cryostat sections of the 23 kidney tissues according to standard procedures [Collan et al., 1991; Kim et al., 1993].

Qualitative immunoperoxidase staining for the presence of hantavirus glyco- and nucleoproteins was performed with formaldehyde fixed, paraffin-embedded sections with specific monoclonal antibodies and carried out essentially as previously described [Groen et al. 1995b]. The mouse monoclonal antibodies (MCAs) selected for this study [Arikawa et al., 1989; Ruo et al., 1991] were directed against the glycoprotein (G2) (MCAs 8E10 and 11E10) and the nucleoprotein (NP) (MCAs GB07 and AA06). Specificities and sensitivities of the respective immunoperoxidase assays, with these MCAs for the detection of the respective proteins in tissues have been evaluated previously [Kim et al., 1993; Groen et al., 1995b]. A rabies virus glycoprotein specific mouse MCA was used as a control in all these assays.

Results

Epidemiology and laboratory findings

Amongst the 13 hantavirus seropositive patients from whom kidney biopsies had been collected, four did not show specific IgA and IgM serum antibody, probably due to the late serum collection (>6 months after the onset). All the other serologically confirmed NE patients with PUUV serum IgG titers ranging from 180 to ≥1620 had also specific serum IgA titers ranging from 20 to 540 and were specific IgM positive (Table 1). In the age and sex matched PUUV seronegative control group no PUUV specific IgA and IgM antibodies were detected (Table 1). There was no significant difference in the prevalence of microhaematuria and proteinuria between the seropositive and seronegative groups. Serum creatinine levels were

higher in the seropositive group as compared to the seronegative group with values of 699 ± 335 and 266 ± 263 µmol/l, respectively (Tables 1 and 2).

Table 2. Results of clinical chemistry, immunohistochemistry and hantavirus specific antigen detection in PUUV seropositive and seronegative patients with renal disease, expressed in percentage (%)

Variable	PUUV seropositive	PUUV seronegative
Clinical chemistry		
Serum creatinine (µmol/l)	699 ± 335*	266 ± 263*
Microheamaturia	77	70
Proteinuria	62	70
Immunohistochemical		
Tubular IgM	15	0
Tubular IgA	0	0
Tubular IgG	0	0
Tubular complement component C3	38	0
Tubular fibrinogen	17	0
Glomerules IgG	0	20
Glomerules IgM	38	50
Glomerules IgA	0	40
Glomerules complement component C3	31	40
Glomerules fibrinogen	0	0
Hantavirus antigen detection		
glycoprotein (G2)	60	20
nucleoprotein (NP)	60	10

* mean ± sd

Histopathological findings

The histopathological findings of kidney biopsies collected from the PUUV seropositive and the seronegative patients are shown in Table 3. Interstitial inflammation was found in all the PUUV seropositive patients and in nine out of ten of the PUUV seronegative patients with mean scores (MS) of 1.5 and 1.3 respectively. Interstitial haemorrhages were exclusively found in the cortex (38% MS 0.4) and medulla (23% MS 0.5) of PUUV seropositive patients. Interstitial edema was found in about 15% and 10% of the individuals of the respective groups. Interstitial hemosiderin deposits were only found in one PUUV seropositive patient (8%, MS 0.1). Interstitial fibrosis was found more frequently in the PUUV

seronegative group (70% MS 1.0) than in the PUUV seropositive group (15% MS 1.0).

In the tubules of the PUUV seropositive and PUUV seronegative groups similar histopathological abnormalities were found. High levels of tubular atrophy were present in both groups (92% to 80%, respectively). Between 46% and 62% of the patients of both groups showed tubular dilation and the presence of hyaline droplets or vacuoles in tubular cells. Tubular mitose was found in 20% of the PUUV seropositive and 30% of the PUUV seronegative patients. Tubular pigment was

Table 3. Renal biopsy findings in PUUV seropositive and seronegative patients, in retrospective confirmatory evaluation.

Histological findings	PUUV seropositive		PUUV seronegative	
	Percentage of patients (%)	Mean score* (degree of severity in no. of biopsies)	Percentage of patients (%)	Mean score* (degree of severity in no. of biopsies)
Interstitium				
inflammation	100	1.3 (1 in 9; 2 in 4)	90	1.5 (0 in 1; 1 in 4; 2 in 4; 3 in 1)
hemorrhage cortex	38	0.4 (0 in 8; 1 in 5)	0	0.0
hemorrhage medulla	23	0.5 (0 in 10; 1 in 1; 2 in 1; 3 in 1)	0	0.0
edema	15	0.2 (0 in 11; 1 in 2)	10	0.1 (0 in 9; 1 in 1)
hemosiderin	8	0.1 (0 in 12; 1 in 1)	0	0.0
fibrosis	15	0.2 (0 in 10; 1 in 3)	70	1.0 (0 in 3; 1 in 4; 2 in 3)
Tubules				
atrophy	92	1.0 (0 in 1; 1 in 11; 2 in 1)	80	1.1 (0 in 2; 1 in 6; 2 in 1; 3 in 1)
dilation	62	0.7 (0 in 5; 1 in 7; 2 in 1)	60	0.8 (0 in 4; 1 in 4; 2 in 2)
hyaline droplets	46	0.5 (0 in 7; 1 in 6)	60	0.6 (0 in 4; 1 in 6)
mitosis	23	0.2 (0 in 10; 1 in 3)	23	0.4 (0 in 6; 1 in 4)
pigment	8	0.1 (0 in 12; 1 in 1)	30	0.3 (0 in 7: 1 in 3)
Glomerules				
hypercellularity	54	0.6 (0 in 6; 1 in 6; 2 in 1)	60	0.6 (0 in 4; 1 in 6)
congestion	15	0.2 (0 in 11; 1 in 2)	30	0.3 (0 in 7; 1 in 3)
Arteriolar changes	30	0.4 (0 in 9; 1 in 3; 2 in 1)	20	0.2 (0 in 8; 1 in 2)
Widening of mesangium	8	0.1 (0 in 12; 1 in 1)	10	0.2 (0 in 9; 2 in 1)

* scale 0-3

found slightly more frequently in the PUUV seronegative group (30%) as compared to the PUUV seropositive group (8%).

No significant differences in histopathological changes in the glomerules were found between both groups. Hypercellularity in glomerules was also a major change

which was found in about 50% of the PUUV seropositive patients (MS 0.6) and in about 60% of the PUUV seronegative patients (MS 0.6). Glomerular congestion, arteriolar changes and enlargement of mesangium were found in 15% (MS 0.2), 30% (MS 0.4) and 8% (MS 0.1) of the PUUV seropositive group, and in 30% (MS 0.3), 20% (MS 0.2) and 10% (MS 0.2) of the PUUV seronegative group.

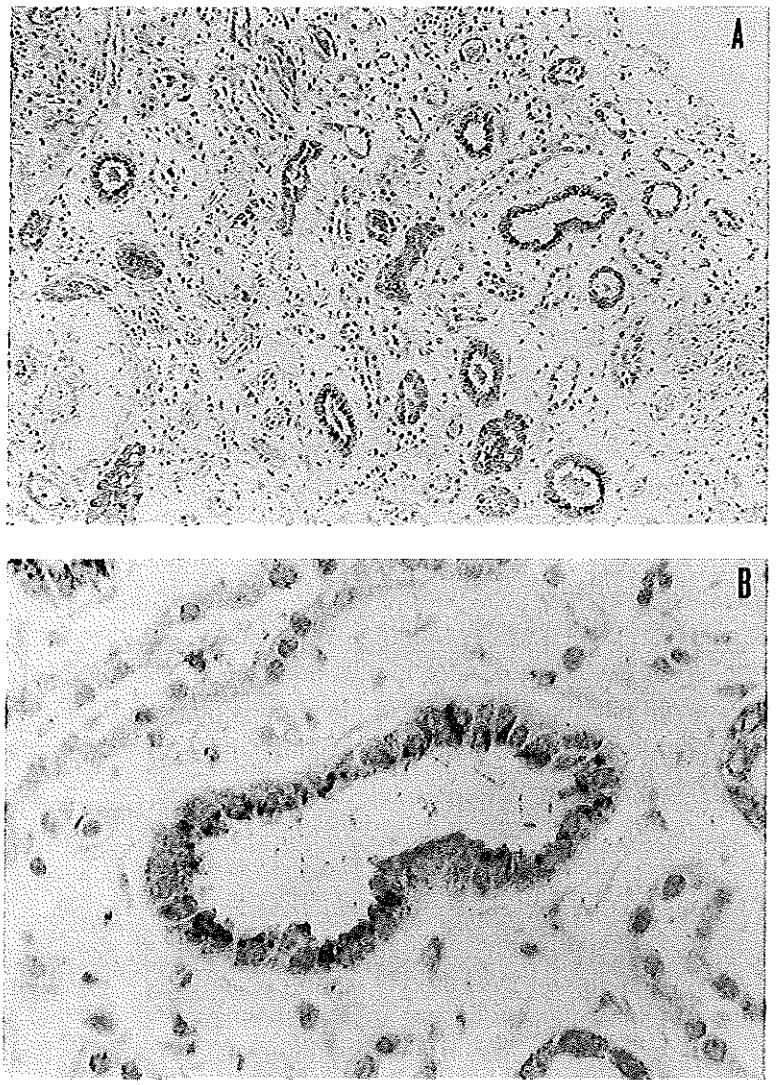

Figure 1. **A**: Cortical area of kidney from PUUV seropositive NE patient with hantavirus specific immunoperoxidase staining in tubular epithelial cells (magn. x200); **B**: Higher magnification showing distinct intracytoplasmic staining in tubular epithelial cells (magn. x400).

Immunohistochemical staining for immunoglobulin deposits

Kidney biopsies of PUUV seropositive and seronegative patients were tested for the presence of deposits of immunoglobulins, complement component C3 and fibrin in the tubules and glomerules by immunofluorescent staining. In the PUUV seropositive patients deposits of IgM, complement component C3 and fibrin were demonstrated in the tubules, along the tubular basement membrane, in 15%, 38% and 17%, respectively. In the PUUV seronegative patients no deposits of Ig, complement component C3 or fibrin were detected in tubules (Table 2). In the glomerules of the seropositive patients deposits of IgM and complement component C3 were detected in the mesangial areas in 38% and 31%, respectively. No deposits of IgA, IgG or fibrin were detected in these patients. Deposits of IgG, IgM, IgA and complement component C3 in the mesangial area were present in the kidneys of the PUUV seronegative patients in 20%, 50%, 40% and 40%, respectively.

Immunoperoxidase staining for the presence of hantavirus antigens

The kidney biopsies from both groups of patients were tested for the presence of glyco- and nucleoprotein antigens by immunoperoxidase staining. In the kidneys from six out of ten PUUV seropositive patients (60%), hantavirus glyco- and nucleoprotein antigen was detected. Remarkably, also in the kidneys from two out of ten PUUV seronegative patients (20%) viral antigen was demonstrated. In one (Table 1, patient 17) both glyco- and nucleoprotein antigens were demonstrated, whereas in the other (Table 1, patient 16) only the presence of glycoprotein could be shown. The distribution of viral antigen was quite similar in the PUUV seropositive and seronegative patients whose kidneys scored positive in this assay: in the cytoplasm of the renal tubular epithelial cells and in the area where the cortex boarders the medulla. The overall distribution pattern of the antigen positive tubules proved to be largely focal (Figures 1A and 1B). With the control MCA no staining was observed in any of the biopsies.

Discussion

In the present study, we have shown the data of a retrospective analysis of kidney biopsies from PUUV seropositive and seronegative patients for the presence of histopathological abnormalties and hantavirus antigen. The biopsies had been taken from patients with renal dysfunction, in a PUUV endemic area in The Netherlands [Groen et al., 1995a]. The most prominent difference between the histopathological findings of both groups was, that interstitial haemorrhages, which were most abundant in the cortical region, were only found in the PUUV seropositive patients.

Although no significant differences in the histopathological findings of the tubules were observed between both groups of patients, tubular abnormalities like sloughing and necrosis of the epithelium accompanied by haemorrhages proved to be important pathological findings in patients with NE. This is in agreement with observations of others in HFRS and NE patients [Cosgriff et al., 1991; Collan et al. 1991]. The degree of histopathological damage in the interstitium, tubules and glomerules was relatively limited in both groups of patients. The mild histopathological findings which we observed are not unexpected for NE which is a mild form of HFRS [Van Ypersele De Strihou and Mery, 1989; Collan et al., 1991; Mustonen et al., 1994]. The same renal tubular abnormalities were observed in the PUUV monkey model that we have recently developed [Groen et al., 1995b]. Interstitial fibrosis, frequently found in the control group but virtually absent in the NE patients is probably linked to more chronical renal disorders which are not found in NE patients. As expected, other findings like tubular atrophy, dilation, vacuolisation and tubular mitoses are not specific for hantavirus nephropathy. The observed non-virus specific renal immunofluorescence findings in the glomerules were scanty and also not restricted to NE patients [Jokinen et al., 1978; Mustonen et al., 1994]. The observed deposits of complement component C3, IgM and fibrin along the tubular basal membrane in the kidneys of the PUUV seropositive patients proved to be indicative for the histopathogenesis of NE.

By immunoperoxidase staining performed with hantavirus specific MCA's, hantavirus antigen was detected in the cytoplasm of renal tubular epithelial cells with an identical localization and distribution pattern between glyco- and nucleoprotein antigens. The characteristic focal distribution of the antigens has been observed before in humans with Hantaanvirus infections and in monkeys with PUUV infections [Kim et al., 1993; Groen et al., 1995b]. The observed presence of viral antigen in the epithelial cells of the tubules indicates that virus replication takes place at this site [Tao et al., 1992; Kim et al., 1993], which may be at the basis of the pathogenesis of acute renal failure in these patients. The fact that in four out of ten seropositive patients no viral antigen could be demonstrated by immunoperoxidase staining in biopsies taken in the acute stage of the infection, may at least in part be due to sampling error as areas with viral antigen are apparently focally spread in the kidney and may be missed by needle biopsies. This may also explain why only in a minority of the NE patients focal haemorrhages were demonstrated.

In the control group two patients with focal sclerosis and granulomatous interstitial nephritis were positive for hantaviral antigen in their kidneys without having developed detectable serum antibodies to PUUV. This observation seems contradictory, but may be in agreement with earlier observations in certain humans and monkeys, who after hantavirus infection, failed to mount significant or persistent antibody response to PUUV [Pether et al., 1993; Groen et al., 1995b]. Non specific

staining is highly unlikely in these two patients, since the MCAs used have been shown to give only specific staining in kidney sections with buffers containing excess of immunoglobulin [Kim et al., 1993]. Furthermore, the pattern and localization of immunoperoxidase staining proved to be identical to that observed in the PUUV seropositive humans and monkeys [Kim et al., 1993; Groen et al., 1995b]. In monkeys we demonstrated that the presence of viral antigen correlates with the presence of viral RNA in the kidneys. Unfortunately, in this study, we could not perform RNA detection assays as performed in the monkeys due to limited availability of human biopsy material. Therefor, further studies of kidney biopsies from more PUUV seropositive and seronegative patients with renal dysfunction in PUUV endemic areas, together with the demonstration of PUUV specific T cell responses in individuals and monkeys without demonstrable PUUV specific serum antibodies could confirm the significance of hantavirus antigen detection in kidneys of patients without hantavirus specific serum antibodies.

Taken together our data show that, the demonstration of PUUV antigen in kidney biopsies of patients suspected of NE, may be an important adjunct to histological and serological findings indicative of a PUUV infection. Retrospective antigen detection in stored kidney biopsy samples from patients with symptoms indicative for NE or more serious forms of HFRS, may prove to be an useful tool to document the prevalence of Hantavirus infections in areas where these have not been identified before.

Acknowledgements

We thank Dr. A. Sanchez and the late Dr. J.M. Dalrymple for providing the monoclonal antibodies, Ms. J. Robinson and Ms. J.B. de Boer for excellent technical assistance, Dr. A.M. Croonen, Dr. H. Hillen and Dr. F.A.TH.W.B. Lustermans for the opportunity to use the biopsied kidney specimens and Ms. C. Kruyssen for help in preparing the manuscript. This work was supported by a grant from the Dutch Veterinary Inspectorate of Public Health.

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CHAPTER 1 2 3 4 5

Epidemiological aspects of Hantavirus infections in The Netherlands

Identification of hantavirus seroptypes by testing of post-infection sera in immunofluorescence and enzyme-linked immunosorbent assays
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Identification of hantavirus serotypes by testing of post-infection sera in immunofluorescence and enzyme-linked immunosorbent assays

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Journal of Medical Virology 1991, 33:26-32

Summary

Serum samples were collected from 27 individuals who had been infected with a member of the genus *Hantavirus* in The Netherlands or Belgium during the last 15 years. These samples were tested in an immunofluorescence assay (IFA) and two enzyme-linked immunosorbent assay (ELISA) systems, using different virus strains that represented each of the four recently proposed serotypes of this genus. The serum samples from 11 individuals who had been infected through contacts with laboratory rats showed the highest reactivities with Hantaan virus (serotype I) and SR-11 (serotype II) in the IFA and ELISA systems. The samples of 16 individuals who had probably been infected through contacts with wild rodents showed the highest reactivities with Hällnäs virus (serotype III) in the IFA. All except two of these also showed the highest reactivity with Hällnäs virus in the two different ELISA systems.

Introduction

Hantavirus (HV) is a newly defined genus of the family *Bunyaviridae*, for which at least four different serotypes have been proposed [Lee et al., 1985b; Goldgaber et al., 1985; Dantes et al., 1987; Lee and Van der Groen, 1989; Sugiyama et al., 1987; Van der Groen et al., 1987]. Members of this genus are transmitted to man by direct or indirect contacts with subclinically infected rodents which serve as a reservoir. Most of these have been associated with serious infections in man, causing haemorrhagic fever with renal syndrome (HFRS). The proposed serotypes are more closely associated with the genus of the reservoir host than with geographical areas: *Apodemus* (serotype 1), *Rattus* (serotype II), *Clethrionomys* (serotype III), and *Microtus* (serotype IV). Serotypes I and II are generally associated with more severe clinical manifestations in man, causing mortality rates up to 10%, whereas serotype

III generally causes milder symptoms and a mortality of less than 1% [Tsai, 1987; Lee and Van der Groen, 1989; Koolen et al., 1989]. Infections with serotype IV have so far not been associated with clinical symptoms in humans [Lee and Van der Groen, 1989; Childs et al., 1987]. Human infections due to contact with wild rodents in Europe are generally caused by serotype III [Van der Groen et al., 1983; Tsai, 1987; Osterhaus et al., 1989]. For the differentiation of HV serotypes, several assay systems using monoclonal and polyclonal antibodies have been used. These include immunofluorescence assay (IFA), plaque reduction neutralization assay, Western blot analysis, enzyme-linked immunosorbent assays (ELISA), precipitation, and haemagglutination inhibition tests [Lee et al., 1985b; Goldgaber et al., 1985; Dantes et al., 1987; Takahashi et al., 1986; Zöller et al., 1989]. For the determination of the serotypes that have infected certain individuals, IFA on convalescent sera has been the most widely used assay. Recently we described different ELISA systems for the serology of HV infections in humans [Groen et al., 1989]. In the present paper we compared two of these assays an indirect ELISA and an inhibition ELISA with an IFA for the determination of serotypes that had been incriminated in recent human cases of HV disease in The Netherlands and Belgium. In both ELISA and IFA systems it was shown that human cases acquired through contacts with laboratory rats were associated with infections with serotypes I or II, whereas in most of the cases in which a contact with wild rodents could be assumed, a closer association with serotype III was found.

Materials and methods

Virus and antigen-preparations

HV, serotype I (strain HV76-118) [Lee et al., 1978], serotype II (strain SR-11) [Kitamura et al., 1983], serotype III (strain Hällnäs) [Yanagihara et al., 1984], and serotype IV (strain Prospect Hill) [Lee et al., 1985a] were propagated in Vero E6 cells. When 80% of the cells were infected, as determined by IFA, the infected cell cultures were solubilized by treatment with 1% Triton X-100 and sonicated twice for 15 seconds as described previously [Groen et al., 1989]. The supernatants were used as antigen after storage at -20°C.

Serum samples

Serum samples were collected from 11 individuals who had been infected with HV after contacts with laboratory rats in the Netherlands or Belgium in 1981. Their clinical histories have been described elsewhere [Desmyter et al., 1983; Osterhaus et al., 1984]. Sixteen serum samples were collected from individuals who had developed HV disease after infection probably due to contact with wild rodents in

The Netherlands or Belgium during the last 15 years as previously documented [Tsai, 1987; Osterhaus et al., 1989; Van der Groen, 1987; Lahaije et al., 1989]. Rodent antisera specific for serotypes I and II were generated by experimental intranasal infection of two groups of six specific pathogen free rats, housed in separate pressurized glove boxes. Six weeks later all the rats were bled by cardiac puncture, their sera were pooled per group and used as reference antisera. For practical reasons, antisera against serotypes III and IV were raised in rabbits. Six New Zealand white laboratory rabbits were infected by a single intramuscular injection of Vero E6 cell propagated Puumala virus (serotype III) or Prospect Hill virus. Animals were bled from the marginal ear vein at three-day intervals from six to eight weeks post-infection and all sera were pooled before dispensing as references reagents.

Indirect Immunofluorescence (IFA)

An IFA was performed, essentially as described previously [Groen et al., 1989]. In brief, infected Vero E6 cells were collected and suspended in growth medium containing 10% (v/v) dimethylsulfoxide at a density of 3.5 x 10^6 cells/ml. After gamma irradiation (2.5 x 10^6 rad), the suspension was stored in 2 ml volumes at -70° C until use. After thawing, the cell suspension was centrifuged for 5 min at 580 x g, the cells were washed three times in PBS, and then resuspended in PBS containing 5% FCS. This suspension was seeded on Teflon-coated drop slides (Wellcome, Beckenham, UK), fixed with -70° C ethanol, and stained with either human, rabbit or rat sera as the first antibody. The second antibody used was a rabbit anti-human, swine anti-rabbit (Dakopatts, Glostrup, Denmark), or goat anti-rat (Nordic, Tilburg, The Netherlands) Ig preparation conjugated with fluorescein isothiocyanate. Results were considered positive when a characteristic dot-like immunofluorescence pattern was observed in the cytoplasm of infected cells. Titers were expressed as the reciprocal of the dilution of primary antibody still giving clear immunofluorescence.

Indirect ELISA

The indirect ELISA was performed in a microtiter system essentially as the cell-ELISA that we described previously [Groen et al., 1989], using the respective virus antigens described above instead of fixed infected cells. In brief, antigen was coated to microtiter plates in phosphate buffered saline (PBS pH 7.2) after determination of the optimal dilution by checkerboard titration using sera from rats and rabbits raised against the different serotypes. After adding a 1:100 diluted human serum samples, plates were incubated for 1 hr at 37°C and binding of the human Ig was detected by adding a sheep anti-human Ig peroxidase conjugate (Amersham International, Amersham, UK). Plates were developed by adding tetramethylbenzidine as a substrate and optical density was measured at 450 nm (OD_{450}). Rat sera were diluted 1:30 and rabbit sera 1:100, goat anti-rat Ig peroxidase (Cappel Cooper Biomedical,

West Chester, PA) and goat anti-rabbit Ig peroxidase (Sigma, St. Louis, MO) were used as conjugate. The results obtained with each serum were expressed as the difference between the OD_{450} obtained with HV antigen and the OD_{450} obtained with control Vero E6 antigen. The OD_{450} of negative human sera (n = 485) never exceeded 0.05.

Complex trapping blocking (CTB) ELISA

A CTB ELISA was performed in a microtiter system as previously described [Groen et al., 1989]. In brief, plates were coated with an optimal dilution of a Protein-A purified IgG fraction of a serum from an individual who had been infected with serotype II [Desmyter et al., 1983; Osterhaus et al., 1984]. Serial twofold dilutions of the sera to be tested were incubated in the plates together with an optimal dilution of serotype II HV antigen. Subsequently the reduction of antigen binding, as detected by a horseradish peroxidase conjugate of the same serum used as a capture antibody, was measured. Antibody titers were expressed as the reciprocal of the highest serum dilution giving at least 40% reduction of the OD_{450} obtained with negative human sera. The same test was used for the titration of antibodies against serotype III, using the IgG fraction of an individual who had been infected with serotype III as capture antibody and conjugate and serotype III HV antigen. The sensitivity, specificity, and reproducibility of the indirect ELISA as well as the CTB ELISA has been described [Groen et al., 1989].

Results

Reactivities of post-infection sera with different HV serotypes in IFA

A comparison of the reactivities in IFA of the sera from individuals who had been infected with HV through contacts with laboratory rats (n = 11) or through contacts with wild rodents (n = 16) and of the used antisera is shown in Figure 1. The antibody titers of the sera from laboratory-associated human cases proved to be about the same against serotypes I and II and generally higher than against serotypes III and IV (Fig. 1a-e). The titers of the sera from the individuals infected through contacts with wild rodents proved to be generally higher against serotype III than against the other serotypes (Fig. 1b,d,f). The serum pool of the rats experimentally infected with serotype I only showed reactivity with type I (titer 20) and not with the other serotypes (Fig. 1d,e,f). The serum pool of the rats experimentally infected with serotype II showed the same titer against serotypes I and II (titer 180), and no reactivities with the other two serotypes (Fig. 1b,d,c,f). The rabbit serum raised against serotype III showed the highest titer (titer 1620) against serotype III (Fig. 1b,d,f), a titer of 540 against serotype IV (Fig. 1c,e,f), and was negative (titer <20)

against the other two serotypes. The rabbit serum raised against serotype IV showed the same reactivities with serotypes II, III, and IV (titer 540) (Fig. 1b,c,d,e,f) and had a titer of 180 against serotype I.

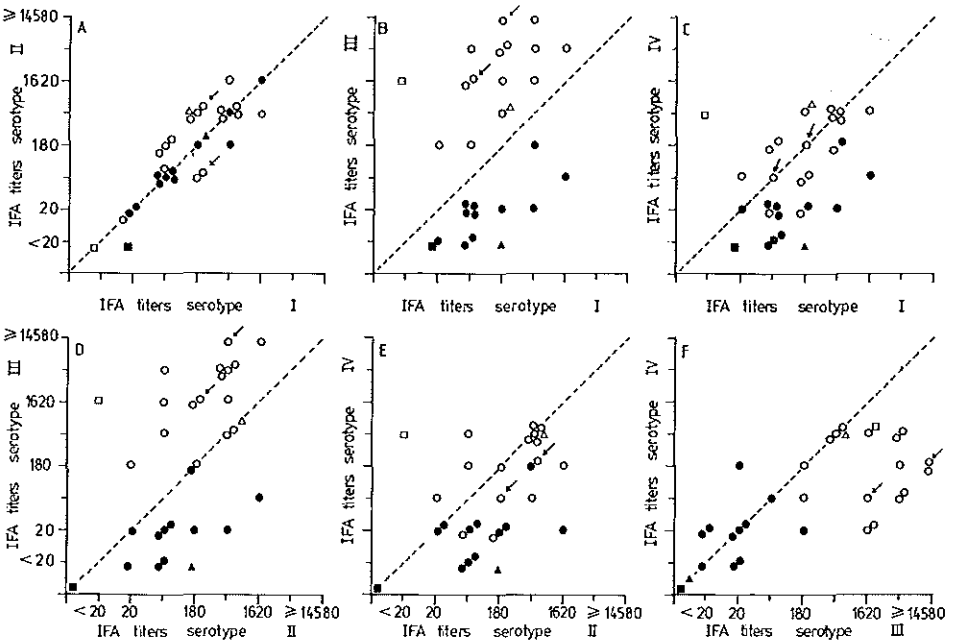

Figure 1. Comparison of IFA antibody titers in sera from laboratory and non-laboratory-association HV infections against four different serotypes of HV ●, sera from laboratory-associated human HV infections; ○, sera from non-laboratory-associated human HV infections; ■, serum pool of laboratory rats infected with serotype I (strain HV76-118); ▲, serum pool of laboratory rats infected with serotype II (strain SR-11);□, serum pool of laboratory rabbit infected with serotype III (strain Puurmala); △, serum from laboratory rabbit infected with serotype IV (Strain Prospect Hill). Arrows: samples showing different patterns of reactivities in IFA and ELISA.

Reactivities of post-infection sera with different HV serotypes in indirect ELISA

Generally the same results were obtained when the same collection of sera tested in the IFA was assayed in the indirect ELISA (Fig. 2). Sera from the laboratoryassociated human cases reacted about the same with serotypes I and II (Fig. 2a,b,c) and generally better than with serotypes III and IV (Fig.2b,c,d,e). Also the reactivities of sera from individuals infected through contact with wild rodents in The Netherlands and Belgium proved to be generally higher against serotype III than against the other serotypes (Fig. 2b,d,f) in this assay. However, two of these sera indicated with arrows in Figure 2 reacted better against serotypes I and II than against serotype III (Fig. 2b,c,d,e). The reactivities of the rat antisera raised against serotypes I and II, respectively, were also higher against both serotypes I and II in this assay than against serotypes III and IV (Fig. 2a,d,e,f). The rabbit anti-serotype III

serum reacted specifically with serotype III antigen. (Fig. 2b,d,f). The reactivity of serum raised in rabbits against serotype IV was only slightly higher against serotype IV than against the other serotypes (Fig. 2d,e,f).

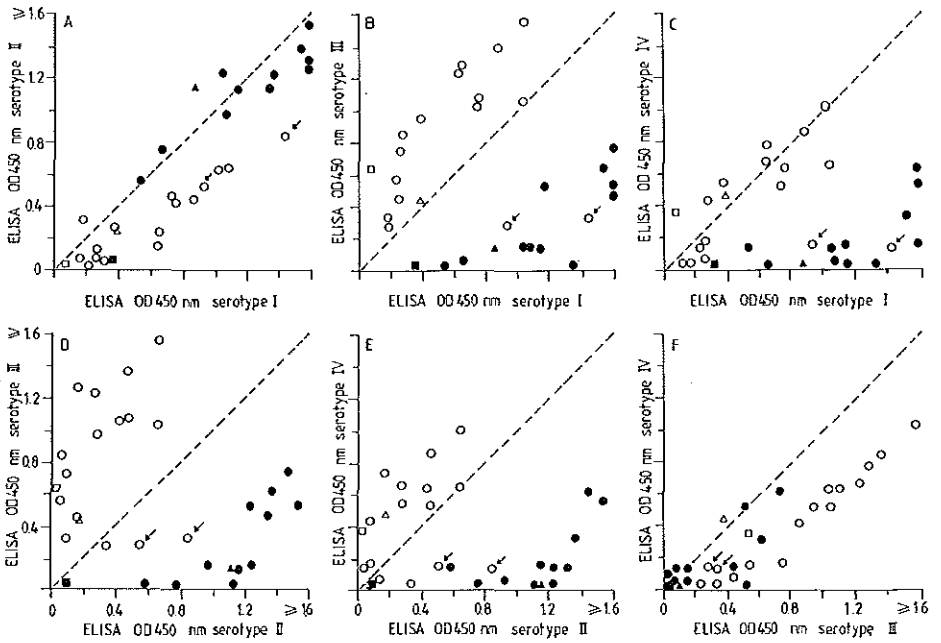

Figure 2. Comparison of indirect ELISA OD $_{450}$ in sera from laboratory- and non-laboratory-associated HV infections against four different serotypes of HV. ●, sera from laboratory-associated human HV infections; ○, sera from non-laboratory-associated human HV infections; ■, sera pool of laboratory rats infected with serotype I (strain HV76-118); ▲, serum pool of laboratory rats infected with serotype II (strain SR-11); □, serum from laboratory rabbit infected with serotype III (strain Puumala); △, serum from laboratory rabbit infected with serotype IV (strain Prospect Hill). Arrows: samples showing different patterns of reactivities in IFA and ELISA.

Reactivities of post-infection sera in CTB ELISA

To further confirm the specificities of the reactivities measured with the human sera in IFA and indirect ELISA with the different virus strains, a third assay system, the CTB ELISA, which is based on a different principle, was examined. Since discrepant values between IFA and the indirect ELISA were found with two human sera, serotypes I and II versus serotype III (see arrows in Fig's 1, 2), CTB ELISA's were carried out with the human sera using serotypes II and III as antigens. The reactivities of the antigens of the different serotypes with the human anti-serotypes II and III IgG preparations were first evaluated in the absence of serum, in order to determine the specificities of these IgG preparations (Fig. 3). Antigens of serotype II IgG preparation than the other two antigens (Fig. 3a), whereas antigen of serotype III reacted better with the anti-type III IgG preparation than the other antigens (Fig. 3b).

Testing of the human sera in the CTB-ELISA against serotypes II and III showed similar results (Fig. 4) as obtained in the indirect ELISA (Fig. 2b): sera from laboratory associated human cases reacted better with serotype II than with serotype III, whereas the reactivities of the sera from non-laboratory associated human cases reacted generally better with serotype III than with serotype II. However, the same two samples of the non-laboratory-associated human cases, which exhibited higher values against serotype II than against serotype III in the indirect ELISA, also showed higher titers against serotype II in the CTB ELISA.

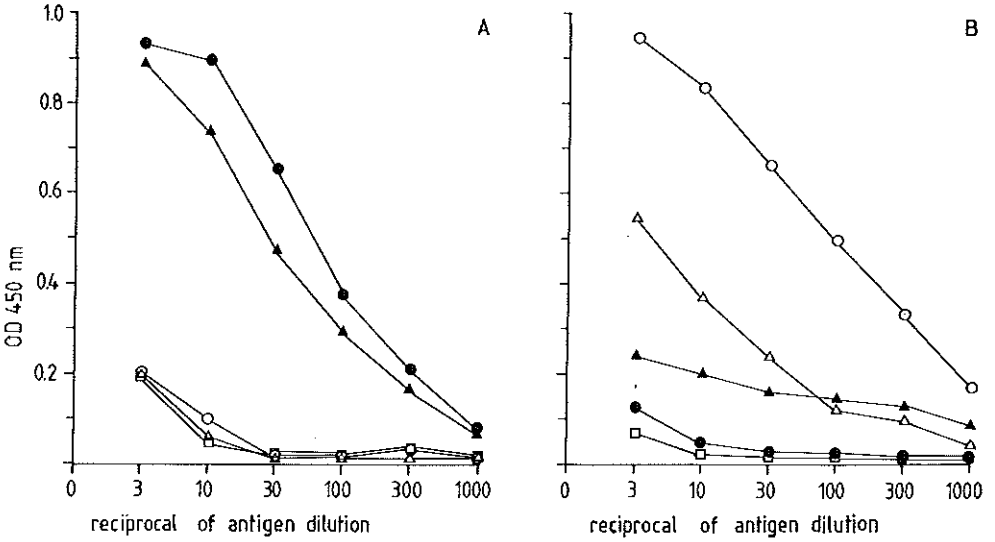

Figure 3. Reactivities in ELISA of IgG prepared from sera from two individuals infected with HV serotype II (A) or HV serotype III (B), used as capture antibody and peroxidase conjugate with antigens of four different HV serotypes. ▲-▲, serotype I (strain HV76-118); ●-●, serotype II (strain Sr-II); ○-○ serotype III (strain Hällnäs); △-△, serotype IV (strain Prospect Hill) and □-□, control Vero E6 antigen.

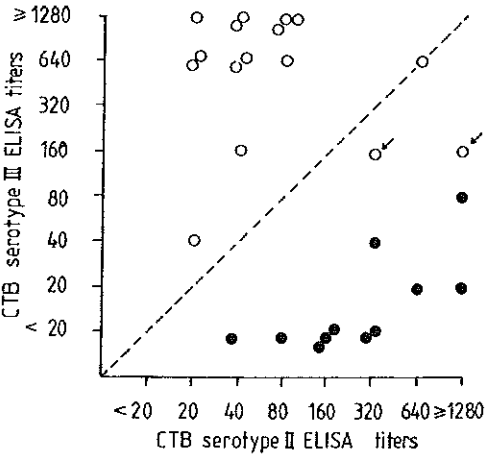

Figure 4. Comparison of CTB ELISA antibody titers of sera from laboratory- and non-laboratory-associated human HV infections against serotype II and serotype III, ●, laboratory-associated human HV infections; ○, non-laboratory-associated HV infections. Arrows: samples showing different patterns of reactivities in IFA and ELISA.

Discussion

In the present study, we have evaluated different serological methods for the retrospective identification of HV serotypes by testing post-infection sera. For this purpose the IFA has been the most generally used assay. Disadvantages of this test include problems with standardization, subjectivity of interpretation, and the laboratory manipulations involved. Comparison of the results of this assay with those of a recently described indirect ELISA [Groen et al., 1989] performed with a collection of human and animal post-infection sera showed that the indirect ELISA gave essentially the same results, with few exceptions. All of the laboratory-associated human infections from The Netherlands and Belgium could be related to infections with serotypes I or II in both assay systems. This reconfirms the nature of these infections [Desmyter et al., 1983; Osterhaus et al., 1984] and also confirms the close antigenic relationship between serotypes I and II. This was also shown by the results obtained with the rat anti-serotypes I and II sera (Figs. 1 and 2) and by showing that antigens of these serotypes reacted better with the anti-serotype II IgG preparation than the other antigens (Fig. 3). In the IFA, all the non-laboratory associated human cases (n = 16) from The Netherlands and Belgium were related to infection with serotype III. this is consistent with earlier observations, indicating that the HV infections in Europe are generally caused by serotype III [Koolen et al., 1989 Tsai, 1987; Osterhaus et al., 1989; Clement and Van der Groen, 1987]. All the human sera from laboratory- and non-laboratory-associated cases of HV infection showed low reactivities with serotype IV in IFA and indirect ELISA. The association with serotype III was also found when the samples were tested in the indirect ELISA, with the exception of sera from two cases that seemed to react better with serotypes I and II. To further investigate the serotype that had infected these two individuals, the human sera were also analysed in a recently described CTB ELISA, based on a different principle [Groen et al., 1989]. Also in this assay the same two samples showed a higher reactivity with serotype II than with serotype III. Whether this discrepancy in reactivities in IFA and ELISA of these two sera indeed reflects differences in the serotypes of the infecting HV must be evaluated with caution. Anamnestic evaluation of their possible infection histories did not indicate that these individuals might have acquired their HV infection outside The Netherlands or Belgium. Also no data about the reservoir rodent species from which they could have acquired the HV infection are available. In this light it is interesting to note that recently in Yugoslavia a high incidence of unusually severe human HV infections has been reported [Lee and Van der Groen, 1989; Avsic-Zupanc et al., 1989; Gligic et al., 1988], which was apparently caused by a serotype closely related to serotype I. Further studies are required to define the HV serotypes circulating in Europe and to determine whether e.g. antigen fixation methods used in different serological assay

systems significantly influence the results obtained in each of the respective tests.

In conclusion it can be stated that non-laboratory-associated HV infection in The Netherlands and Belgium are generally caused by infection with serotype III and that the indirect ELISA described is a useful tool for the serological identification of HV serotypes using post-infection human sera. Clear advantages of this assay over the IFA are that it can easily be incorporated in automated screening systems, can be evaluated objectively, and is easy to standardize.

Acknowledgments

We kindly acknowledge Mr. A. Lefévre and Mr. G. Hoofd for technical assistance, and Ms. C. Kruyssen and Ms. M.C. Eskens for help in preparing the manuscript.

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Different hantavirus serotypes in Western Europe

Jan Groen, Albert D.M.E. Osterhaus, Tantjana Avsic-Zupanc, Guido van der Groen, Jan P. Clement, Alex Lefévre and Hein G.M. Jordans.
Lancet 1991, 337:621-622

Sir,- So far, human infections with hantavirus, causing haemorrhagic fever with renal syndrome (HFRS), other than those transmitted by laboratory animals, have in Western Europe been associated with serotype III [Editorial, 1990; Groen et al., 1991]. The main reservoir host is the red bank vole *Clethrionomys glareolus*. In The Netherlands we have found serological evidence for serotype III in people who were probably infected via contact with wild rodents [Osterhaus et al., 1989]. We have now demonstrated this virus in animals. In lung extracts of four seropositive red bank voles caught in an area of The Netherlands where eight confirmed human cases of HFRS had been documented, viral antigen was identified by ELISA. The ELISA identified the virus as serotype III [Groen et al., 1991].

With the same ELISA system we have tested 32 immunofluorescent antibody positive sera, collected from people with a non-laboratory-associated infection in Yugoslavia, against serotype I (strain HV 76-118) which strongly cross-reacts with serotype II (SR-11) [Groen et al., 1991], serotype III (strain Hällnäs), and a recent isolate from Yugoslavia (strain Dobrava) [Avsic-Zupanc et al., 1988]. The Dobrava strain was isolated from the yellow-necked field mouse (*Apodemus flavicollis*) during a recent period of high incidence of HFRS in Yugoslavia [Avsic-Zupanc et al., 1988], it reacted more strongly with sera specific for serotypes I and II than with sera specific for serotype III. In addition 16 positive sera from non-laboratory-associated infections and 11 positive sera from laboratory-associated infections in The Netherlands and Belgium were tested in the ELISA against these three viruses. All sera from the laboratory-associated infections showed higher titers against serotype I and strain Dobrava than against serotype III (Figure, A). The titers of these sera against serotype I were slightly higher than those against strain Dobrava. All but two of the 16 sera from non-laboratory-associated infections in The Netherlands and Belgium showed the highest titers against hantavirus serotype III (Figure, B). 14 of the 32 sera from Yugoslavia showed the highest titers against serotype I and strain Dobrava,whereas the other 18 reacted more strongly against serotype III (Figure, C). This confirms the results of an earlier serological study with an IFA, which indicated that two different hantavirus serotypes caused HFRS in Yugoslavia [Avsic-Zupanc et al., 1989].

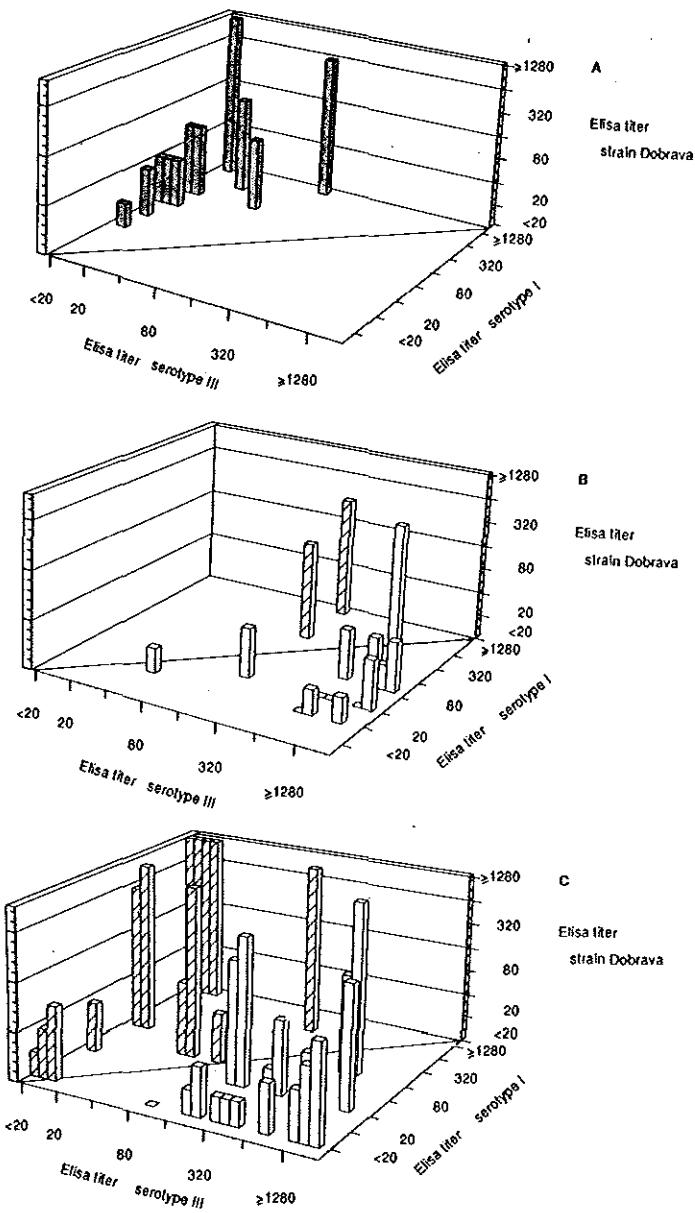

Figure 1. Antibody titers of human sera in ELISA, measured against hantavirus serotype I, serotype III, and strain Dobrava. (A) 11 sera from laboratory-associated cases; (B) 16 sera from non-laboartory associated cases in The Netherlands and Belgium; (C) 32 sera from non-laboratory associated cases in Yugoslavia.

One serum from The Netherlands and one from Belgium reacted more strongly with serotype I and strain Dobrava than with serotype III. Furthermore, both sera

neutralised serotype I (strain HV 76-118) in an 80% plaque reduction neutralisation assay, with titers of 10 and 40, respectively (Jim LeDuc, USAMRIID, Frederick, USA, personal communication). Both individuals had recently been infected and had not recently been abroad. In a report by Pilaski and co-workers, one out of 21 sera from HFRS patients in Germany proved to react more strongly with serotype I [Pilaski et al., 1991]. These data suggest that in Western Europe as in Yugosalvia non-laboratory-associated hantavirus infections may be caused by more than one serotype.

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Hantavirus nephropathy in The Netherlands

Albert D.M.E. Osterhaus, Jan Groen, Fons G.C.M. UytdeHaag , Gijsbert van Steenis, Guido van der Groen, Jan Clement and Hein G.M. Jordans
The Lancet 1989, ii:338-339

Sir,- In 1984 we reported the first cases of hantavirus (HV) infection in The Netherlands [Osterhaus et al., 1984]. HV-specific antibodies were demonstrated by immunofluorescence assay (IFA) in four laboratory workers at the National Institute of Public Health and Environmental Protection (RIVM), who had handled HV-infected Lou/M laboratory rats. One worker had severe transient nephropathy, the other three had only mild symptoms. No HV-specific antibodies were demonstrated by IFA in RIVM laboratory workers who had had no contact with these rats.

Table 1. Samples with hantavirus-specific antibodies* in The Netherlands (1974-89)

Origin	Years	Total	No. positive
Laboratory workers in contact with infected rats*	1981-84	9	4
Workers not in contact with rats	1981-88	171	0
Suspected leptospirosis	1984-89	865	5
Farmers of 82 farms in Friesland	1986-87	488	0
History of severe transient nephropathy 1974-88	1989†	10	7

* ELISA confirmed by IFA and western blot

† Time of serum collection

Using a newly developed ELISA, which permits the rapid screening of large numbers of sera [Groen et al., 1989], we have now tested samples collected from people with different clinical histories in The Netherlands, who had not been in direct contact with laboratory animals (Table 1). In a panel of 865 arbitrarily chosen samples of sera from patients with suspected leptospirosis who proved to be seronegative for leptospira, the samples being collected from all over the country between 1984 and 1988, five ELISA positive sera were found. Testing by ELISA of recently collected samples from 10 individuals with a history of severe transient nephropathy admitted to hospital in the past 15 years, revealed that seven had HV-specific antibodies. one had had nephropathy as early as 1974. The 12 ELISA positive reactions (titers 100 to 20,000) found in these two groups were all confirmed

by IFA and western blot [Groen et al., 1989]. 488 serum samples from farmers living in the northern, non-forested part of the country (Friesland) [Bokhout et al., 1989] and of 171 RIVM workers who had not been in contact with HV-infected laboratory animals, were also screened by ELISA. All sera were negative.

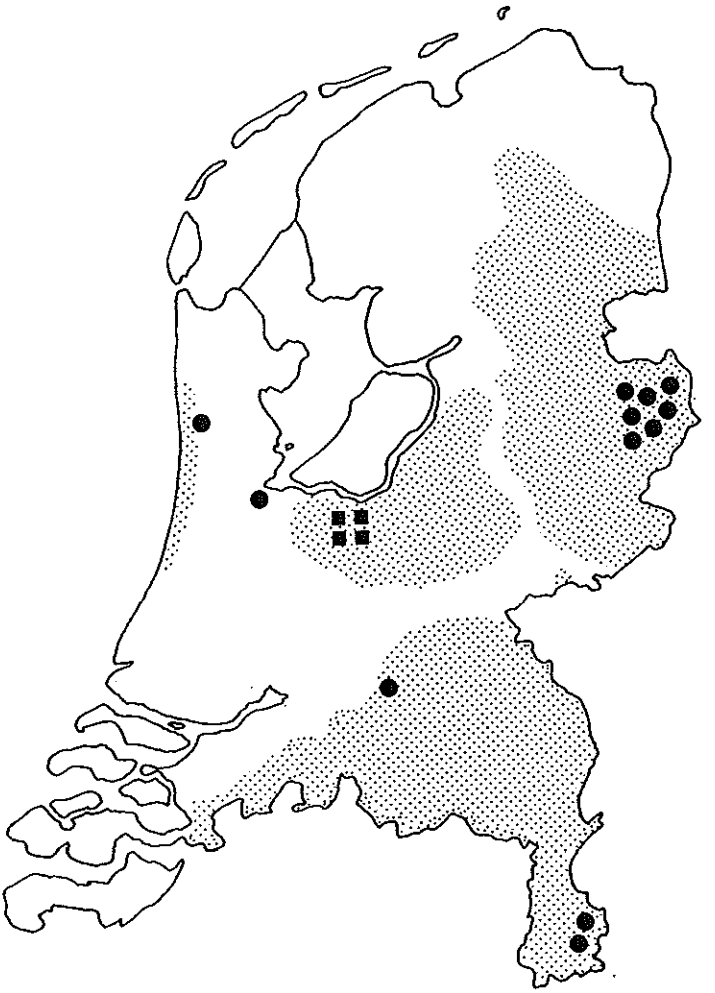

Figure 1. Map of The Netherlands, indicating locations of patients with HV-specific antibodies: ■ laboratory associated cases, ● non-laboratory associated cases, ⊡ forested areas.

Both by ELISA and IFA the four sera from laboratory-associated HV-infected individuals consistently showed at least two-fold higher titers with the HV serotype I ("*Apodemus associated*") strain Hantaan virus 76-118, than with members of the other three proposed serotypes [Lee et al., 1985; Zhang et al., 1989]. The serum samples of the 13 non-laboratory-associated cases of HV infection showed at least

twofold higher antibody titers with serotype III ("*Clethrionomys associated*") strain Hällnäs, than with members of the other proposed serotypes [Groen et al., 1989].

These data suggest that apart from laboratory-associated infections with HV serotype I, infections with HV seroptype III associated with severe renal disease, have occurred in The Netherlands in the past 15 years. Since 10 out of 12 of these seropositive individuals lived in the eastern and southern forested areas of the country (Figure 1) and since at least one of the other two visited one of these areas shortly before the symptoms developed, it is most likely that, as in neighbouring countries West Germany and Belgium [Verhagen et al., 1986; Zeier et al., 1987], wild rodents are the reservoir of the virus.

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Haemorrhagic fever with renal syndrome

Martin N. Gerding, Hein G.M. Jordans, Jan Groen, Albert D.M.E. Osterhaus
Lancet 1993, 342:495

Sir,- Kulzer and colleagues (July 31, p 313) report a substantial rise in the incidence of nephropathia epidemica in Germany during the first half of 1993. In 1989 we reported the increased recognition of hantavirus infection in The Netherlands [Osterhaus et al., 1989]. Each year a few sporadic cases have been diagnosed, mostly in the eastern part of the country. In our hospital this amounted to 14 patients from 1974 to 1992. In 1993, eight cases of hantavirus induced acute renal failure have been treated in our hospital, seven since June.

In this area, near the German border, an abundance of bank voles (*Clethrionomys glareolus*) has been noted since the spring of this year. Scandinavian experience has shown a relation between the incidence of nephropathia epidemica and the cyclic variation in bank-vole prevalence [Settergren et al., 1988]. We agree with Kulzer et al that the diagnosis of this disease can be easily overlooked and that subclinical cases almost certainly occur. The suggestion of an unrecognised pandemic needs serious attention, because not every case of nephropathia epidemica runs a benign course [Forslund et al., 1992].

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Risk of infections transmitted by arthropods and rodents in forestry workers

Anton W. Moll van Charante, Jan Groen and Albert D.M.E. Osterhaus
European Journal of Epidemiology 1994, 10:349-351

Summary

One hundred and fifty-one forestry workers and 151 matched office clerks were compared as to the presence of antibodies against *Borrelia burgdorferi*, tick-borne encephalitis virus, Puumala virus and lymphocytic choriomeningitis virus. Their occupational risks of being infected by *Borrelia* was fourfold and significant, by Puumala virus and lymphocytic choriomeningitis virus was increased but not significant. No seropositivity has been established against tick-borne encephalitis virus.

Introduction

The medical community is becoming increasingly interested in infectious diseases which are difficult to detect by ordinary clinical investigation, such as tick-borne encephalitis (TBE), haemorrhagic fever with renal syndrome (HFRS), and lymphocytic choriomeningitis (LCM), or which incidentally are difficult to distinguish from other clinical entities, such as lyme borreliosis (LB). Recent studies indicate that these four diseases are widely distributed throughout the world [Rahn et al., 1991; Holmgren et al., 1990; Groen et al., 1991; Editorial, 1992; Nuti et al., 1993]. In fact they are the most important infectious diseases transmitted to humans by arthropods and rodents in the forested areas in Western Europe.

LB, a spirochetosis caused by *Borrelia burgdorferi*, is a tick-borne with small rodents as reservoir - as is TBE, which is caused by a member of the *Flaviviridae* family [WHO, 1986; Schwartz et al., 1990]. In Europe both are mostly transmitted through bites by ticks (*Ixodes ricinus*).

HFRS, caused by the Puumala virus (PUUV) of the genus *Hantavirus*, a member of the *Bunyaviridae* family [Osterhaus et al., 1989; Groen et al., 1991], and LCM, which is caused by lymphocytic choriomeningitis virus, a member of the *Arenaviridae* family are transmitted through direct or indirect contacts wild rodents [Childs et al., 1992].

Individuals who are professionally involved in forestry work and those who live in

forested areas may be expected to be at increased risk of acquiring these infections. A number of studies have been devoted to such populations [Guy et al., 1989; Stanford et al., 1990; Niklasson et al., 1992; Clement et al., 1992]. However, they lacked the use of a matched control group. Therefore, we report here on the comparison of a sample of forestry workers with a matched control group with regard to the prevalence of seropositivity against these four infectious diseases.

Materials and methods

Dutch forestry workers employed in the maintenance of state-owned woodland, heathland and national parks are public employees. This population (n = 750) receives occupational medical care from the State Occupational Health Service. A cohort of 151 of these forestry workers, employed at six of the 23 forestry stations (20%), were screened for markers of these infectious diseases in April 1989 and April 1990. The first screening also included a control group of office clerks, also civil servants categorically matched to the 151 participating forestry workers by age (same decennium) and occupational health centre as a proxy variable for area of domicile.

Serum samples were stored after collection at -20 °C until use. The commercially available flagellum enzyme-linked immunosorbent assay (ELISA) (K416, Dakopatts, Glostrup, Denmark) was used for the detection of IgG directed against *Borrelia burgdorferi* and used as described by the manufacturer. Sera with optical density (OD) value above the cutoff of 0.40 were considered positive after confirmation by Western blot [Hansen et al., 1989; Ma et al., 1992].

TBEV-specific antibodies were measured using the commercially available indirect FSME ELISA (Virotech, Heidelberg, Germany).

For the detection of Hantavirus-specific IgG antibodies the indirect ELISA was used as described elsewhere [Groen et al., 1991]. Hantavirus-positive ELISA reactions were captured by indirect immunofluoresence assay (IFA).

LCMV-specific antibodies were measured by indirect ELISA. The viral and control antigen were prepared from ATCC VR134 virus-infected and uninfected BHK cells. Five days after infection medium was removed and the cells were washed with phosphate buffered saline, pH 7.2 (PBS), trypsinised, washed with PBS plus 10% FCS and centrifuged for 5 min at 580g. The cell pellet was resuspended in 1 ml PBS and sonicated twice for 15 seconds. Cellular debris was removed by low-speed centrifugation. The supernatant was inactivated with 1% ethylene oxide and used as antigen after storage at -70 °C. The LCM virus IgG-ELISA was performed as described for Hantavirus-specific antibodies [Groen et al., 1991]. The indirect IFA was used to confirm the OD values higher than 0.2 above the control antigen level,

using drop slides with LCM virus-infected and uninfected BHK cells fixed with 70% ethanol and stored at -70°C until use.

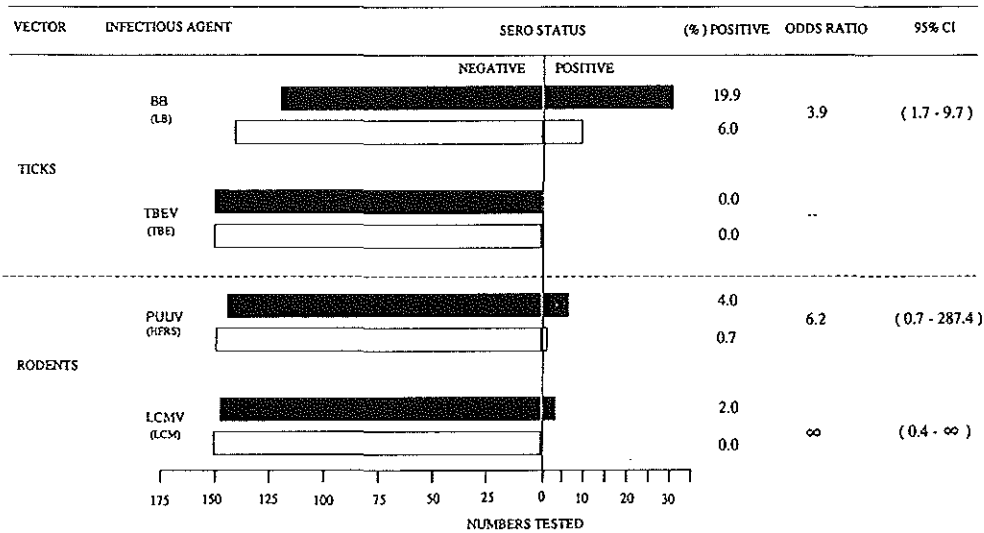

Figure 1. Seroprevalence of arthropod and rodent transmitted infections in forestry workers in the Netherlands. ■ forestry workers; □ non-forestry workers.

Results and discussion

The results of the screening for tick-transmitted infections show that 30 (19.9%) of the forestry workers versus nine (6.0%) of the clerks (odds ratio 3.9, exact estimation) had serum antibodies to *Borrelia burgdorferi* [Moll van Charante et al., 1989], a statistically significant difference, whereas no serum antibodies against TBE were found in either group (Fig. 1). The results of the screening for serum antibodies to the viruses transmitted through contacts with rodents show that six (4.0%) forestry workers had serum antibodies to PUUV versus one (0.7%) of the clerks. It is interesting to note that four of the PUUV-seropositive individuals worked in areas where 14 clinical cases of HFRS had been diagnosed before [Osterhaus et al., 1989; Jordans et al., 1991]. Three (2.0%) forestry workers had serum antibodies to LCMV versus none (0.0%) of the clerks.

A hundred and fifty-one forestry workers were sampled for the second time in April 1990. Five of them, who had no serum antibodies to LB in 1989, were seropositive in 1990 [Schmutzhard et al., 1988]. None of these five individuals had developed clinical symptoms suggestive of LB during the period between the two samplings [Moll van Charante et al., 1989; Jordans et al., 1991]. No seroconversions

towards TBE, PUUV and LCMV were found in the 151 individuals.

Taken together, our data suggest that forestry workers in The Netherlands have an increased risk of LB. Their occupational hazards to PUUV and LCMV infections however, remain to be corroborated.

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Hantavirus infections in The Netherlands: epidemiology and disease

Jan Groen, Martin H. Gerding, Hein G.M. Jordans, Jan P. Clement, Jan H.M. Nieuwenhuijs and Albert D.M.E. Osterhaus
Epidemiology and Infection 1995, 144:373-383

Summary

A serological survey for the prevalence of hantavirus infections in The Netherlands was carried out on >10000 sera, from selected human populations, and different feral and domestic animal species. Hantavirus-specific antibodies were found in about 1% of patients suspected of acute leptospirosis, 10% of patients with acute nephropathia, and in less than 0.1% haemodialysis and renal transplant patients. Among individuals with a suspected occupational risk, 6% of the animal trappers, 4% of the forestry workers, 2% of the laboratory workers and 0.4% of the farmers were seropositive. The majority of the seropositive individuals lived in rural and forested areas. The main animal reservoir of the infection was shown to be the red bank vole (*Clethrionomys glareolus*) Epidemiological, clinical and laboratory findings seen in serologically confirmed human cases were similar to those associated with nephropathia epidemica.

Introduction

The severity of haemorrhagic fever with renal syndrome (HFRS), caused by members of the genus *Hantavirus* (HV) of the family *Bunyaviridae*, is largely dependent on the serotype of the virus involved. So far at least four different subtypes of HV have been characterized [Lee et al., 1985; Sugiyama et al., 1987]. These subtypes are all closely associated with the genus of the reservoir host involved: *Apodemus* - Hantaan-like viruses; *Rattus* - Seoul-like viruses; *Clethrionomys* - Puumala-like viruses and *Microtus* - Prospect-Hill-like viruses. Recently a HV has been identified in the USA as the cause of a severe respiratory disease in humans with high mortality. This infection is transmitted to humans by the deer mouse (*Peromycus maniculatus*) [Childs and Rollin, 1994]. Infections with Hantaan-like, Seoul-like and Puumala-like viruses may cause renal failure in humans and are predominantly transmitted by the air-borne route. The clinical symptoms related to Hantaan- and Seoul-like virus infections are generally characteristic, whereas the symptoms associated with Puumala-like virus infections are more

variable and often difficult to define as belonging to a disease entity [Pether and Lloyd, 1993]. As impairment of renal function of varying severity is a consistent feature of infection with Puumala-like viruses, the syndrome is referred to as nephropathia epidemica (NE). Seroepidemiological studies in Asia, Europe and the USA have demonstrated HV specific antibodies in the sera of individuals, such as farmers and army personnel, at high risk of contact with feral rodents [Osterhaus et a., 1984, 1989; Nuti et al., 1990, 1993; Yanagihara, 1990; Jordans et al., 1991]. Recently, serological evidence of HV infection was also found in patients with chronic renal failure, haemodialysis patients, and in renal transplant candidates [Glass et al., 1990, 1993; Tsianos et al., 1993]. Furthermore, HV-specific serum antibodies have been found in individuals with acute respiratory disease and in patients suspected of acute leptospirosis [Osterhaus et al., 1989; Nuti et al., 1990; Chillds and Rollin, 1994]. The geographical distribution of HV infection in humans is usually focal probably due to the distribution of infected reservoir animals [Yanagihara, 1990]. Although HV has been detected in a large variety of feral and domestic animals [Clement et al., 1994] the main reservoirs are rodent species.

In 1984 we documented the first cases of HV infections in The Netherlands among laboratory workers of the National Institute of Public Health and Environmental Protection (RIVM), who had been in contact with infected laboratory Lou/M rats [Osterhaus et al., 1984]. One of the laboratory workers suffered severe transient renal failure whereas the others showrd milder clinical symptoms. Subsequently, a number of serologically - confirmed human cases of NE, not related to contacts with laboratory animals, were found in the eastern and southern parts of The Netherlands [Osterhaus et al., 1989; Jordans et al., 1991]. In these areas we also identified HV infected feral rodents [Groen et al., 1991a]. We subsequently, showed that the laboratory workers of the RIVM had been infected with Seoul-like viruses whereas the majority of the other seropositive human cases had been infected with Puumala-like viruses [Groen et al., 1991a, 1991b].

In the present study we have investigated the geographical distribution of HV-specific serum antibodies in individuals in The Netherlands with or without a history of renal disease or suspected occupational risk. In addition, sera from a large collection of feral and domestic animals in The Netherlands were tested. Clinical and laboratory findings of the individuals with a history of serologically confirmed NE infection are presented.

Materials and methods

Human serum samples

Human serum samples were collected between 1972 and 1994 from 1783

individuals with renal diseases, including patients with suspected acute leptospirosis, established renal disease, haemodialysis and kidney transplant patients. Sera from 2172 individuals with suspected occupational risk of HV infection (laboratory workers, farmers, hunters, forestry workers, veterinary surgeons, zoologists, gardeners, trappers and military recruits) and 4474 sera from a control group consisting of office personnel and healthy blood donors in apparently enzootic areas (see below) were also collected. Samples were also collected from 463 military office personnel from all over the country. All sera were heat inactivated for 30 min at 56°C directly after collection and stored at -20° C until used.

Feral animal serum and organ samples

Feral animal samples were collected between 1984 and 1993 from 829 animals from 20 different species. Rodents were trapped between 1987 and 1993 at several places in The Netherlands, around houses surrounded by forests and fields, and on camp sites. The rodent species were identified, blood was collected and lung suspensions were prepared (for survey see Table 1). A separate group of rats was obtained from Rotterdam Harbour from an animal pest control service. Bats suspected of rabies were obtained from the Central Veterinary Institute in Lelystad. Serum samples from other feral mammals (Table 1; hare, roe, fox and wild boar) were collected between 1992 and 1993 during the hunting season.

Domestic animal serum samples

Serum samples were collected from 2025 domestic animals representing 14 species at several locations in The Netherlands between 1984 and 1992. Sera from lagomorphs and rodents (Table 1) were obtained from a veterinary clinic, where the animals had been admitted with a variety of clinical symptoms. Serum samples from dogs with a history of acute gastrointestinal disease and from cats routinely examined for feline leukaemia virus infection were collected through veterinary practitioners throughout the country. Sera from cows, sheep and pigs were collected from different slaughter houses.

Serology and antigen detection

Hantavirus-specific antibodies against Puumala-like virus (strain Hällnäs) were detected by a previously described indirect enzyme-linked immunosorbent assay (ELISA) [Groen et al., 1991b]. For the detection of antibodies of species for which no species-specific conjugates were available (members of the families Talpidae, Sciuridae, Cricetidae, Castoridae, Chinchillidae, Caviidae, Capromyidae, Vespertilionidae and Dasyproctidae) a protein-A peroxidase conjugate (Amersham International, Amersham, UK) was used. The specificity and sensitivity of this conjugate for immunoglobulines of different animal species had been evaluated

previously [Kelly et al., 1993]. OD 450 values obtained by subtracting the OD 450 values of control antigen-coated wells from corresponding viral antigen coated wells. Values >0.2 were tested in a confirmatory indirect immunofluorescence test (IFA) at a 1/16 dilution, using drop slides fixed with Vero E6 cells infected with HV strain Hällnäs [Groen et al., 1991b]. Results were considered positive when a characteristic dot-like immunofluorescence pattern was observed in the cytoplasm of infected cells.

HV antigen in lung tissues of feral animals was detected by, a previously described antigen capturing ELISA [Groen et al., 1991b]. For this purpose 10% lung suspensions were prepared in a glass grinder in phosphate buffered saline (PBS) containing 1% Triton X-100 and used as antigen.

Clinical and laboratory findings

The medical records of the 27 hospitalized and seropositive NE patients obtained from three hospitals in The Netherlands were reviewed for clinical chemistry, haematological, clinical findings and epidemiological data. These data were compared with the published findings of NE cases in neighbouring countries [Pilaski et al., 1991; Van Ypersele de Strihou, 1991].

Results

Prevalence of HV specific antibodies in human sera

Between 1972 and 1994, 8892 serum samples from 1783 patients with renal disease, 2172 individuals with suspected occupational risk of HV infection and from 4937 individuals without apparent symptoms of renal disease or risk of HV infection were tested for the presence of HV-specific serum antibodies. In the first group 33/1783 patients (2%) were HV seropositive (Table 2). No HV-specific serum antibodies were found in 284 renal transplant patients and only 1/358 haemodialysis patient (0.3%) was seropositive. Five of the 865 leptospirosis suspected individuals (0.7%) were seropositive. All the seven serum samples collected retrospectively from patients who had been diagnosed with NE on clinical grounds between 1974 and 1988 were seropositive. In the years between 1989 and 1992 HV seropositivity in clinically suspected NE cases ranged from 4% to 11% (Table 2). In the period January - June 1993 7/33 suspected NE cases (21%) were HV seropositive. The geographical distribution of all the clinically documented and serologically confirmed cases is shown in Figure 1.

Among the 2172 individuals with suspected occupational risk of HV infection, HV-specific serum antibodies were found in 4/180 laboratory workers (2.2%) who had worked with contaminated laboratory rodents, in 3/679 farmers (0.4%),

Table 1. Prevalence of HV specific serum antibodies in feral and domestic aimals in The Netherlands.

Group	Order	Family	Species	Common name	Year	positive/ numbers tested	%
Feral	Insectivora	Talpidae	*Talpa europa*	Common mole	89	0/33	0
		Soricidae	*Crocidura russula*	Common shrew	89	1/66*	1.5
	Rodentia	Cricetidae	*Ondata zibethia*	Muskrat	89	0/192	0
			Microtus arvalis	Field vole	89	1/68	1.5
			Clethrionomys glareolus	Red bank vole	84-89	12/111†	10.8
		Muridae	*Apodemus sylvaticus*	Wood mice	89	0/56	0
			Rattus norvegicus	Norway rat	89	0/12	0
			Rattus rattus	Black rat	89	0/4	0
			Mus musculus	House mouse	89	0/12	0
	Lagomorpha	Leporidae	*Lepus capensis*	Hare	93	0/63	0
	Carnivora	Canidae	*Vulpes vulpes*	Red fox	93	0/62	0
		Mustelidae	*Meles meles*	Badger	93	0/3	0
	Artiodactyla	Bovidae	*Capreolus capreolus*	Roe	92	0/80	0
		Suidae	*Sus scrofa*	Wild boar	92	0/10	0
	Chiroptera	Vespertilionidae	*Pipistrellus pipistrellus*	Pipistrelle bat	93	0/31	0
			Epitesicus serotinus	Common brown bat	93	0/17	0
			Plecotus auritus	Common long-eared bat	93	0/6	0
			Pipistrellus nathusii	Pipistrelle bat	93	0/1	0
			Nyctalus noctula	red bat	93	0/1	0
			Myotis dasycnette	Pond bat	93	0/1	0
Domestic	Lagomorpha	Leporidae	*Orytolagus cuniculus*	European rabbit	92	0/113	0
	Rodentia	Sciuridae	*Sciurus carolinensis*	Grey squirrel	92	0/1	0
		Cricetidae	*Cricetus cricetus*	Hamster	92	0/2	0
		Castoridae	*Castor castoridae*	Beaver	92	0/4	0
		Chinchillidae	*Chinchilla laniger*	Chinchilla	92	0/14	0
			Dolichotis patagona	Mara	92	0/3	0
		Caviidae	*Cavia porcellus*	Guinea pig	92	0/59	0
		Capromyidae	*Mycocastor coypus*	Nutria	92	0/2	0
		Dasyproctidae	*Myoprocta acouchy*	Agouti	92	0/1	0
	Carnivora	Canidae	*Canis familiaris*	Dog	91	0/585	0
		Felidae	*Felis caris*	Cat	84	0/200	0
	Artiodactyla	Bovidae	*Bos taurus*	Cow	91	0/579	0
			Ovis aries	Sheep	91	0/254	0
		Suidae	*Sus scrofa*	Pig	84	0/208	0
Total			34 species		84-93	14/2854	0.4

* animal HV antigen positive and HV antibody negative
† Two of the positive animals: no serum available and tested for HV antigen

Table 2. Prevalence of hantavirus specific serum antibodies in selected human populations in The Netherlands

Selected groups	Individuals	year	number	positive	%
renal disease	kidney transplants	72-84	284	0	0
	suspected acute leptospirosis	84-89	865	5	0.7
	suspected NE	74-88*	7	7	100
		89	27	3	11.1
		90	60	3	5.0
		91	81	4	4.9
		92	68	3	4.4
		93	33	7	21.2
	haemodialysis	93	358	1	0.3
suspected occupational risk	laboratory workers	81-84	180	4	2.2
	farmers	87-93	679	3	0.4
	hunters	89	455	0	0
	foresters	89-90	151	6	4.0
	zoologists	91	17	0	0
	trappers	91-93	68	4	5.8
	military	92	460	0	0
	gardeners	92	60	0	0
	veterinary surgeons	93	102	0	0
control	office personnel†	89-90	151	1	0.7
	healthy blood donors†	92	4323	32	0.7
	army office personel‡	92	463	0	0
Total		74-93	8892	83	0.9

* retrospective
† sera collected in endemic areas
‡ sera collected at different barracks all over the country

4/68 trappers (6%) and in 6/151 sera from forestry workers (4%). No HV-specific antibodies were found in hunters, zoologists, military personnel, gardeners or veterinarians. The geographical distribution of HV seropositive laboratory workers, farmers, animal trappers and forestry workers is indicated in Figure 1. Of the 4474 serum samples from individuals in the control groups collected in apparently endemic areas between 1989 and 1992, 33 (0.7%) had HV-specific serum antibodies. No antibodies were found in 151 sera from army office personnel collected at several locations in The Netherlands.

Prevalence of HV specific antibodies or HV antigen in animal samples

Sera from 2855 feral and domestic animals collected between 1984 and 1994 were tested for the presence of HV antibodies. Among 829 sera from feral animals (Table 1) representing 20 different species, HV-specific antibodies were detected in 10/111 (9%) red bank voles (*Clethrionomys glareolus*) and in 1/68 (1%) field voles (*Microtus arvalis*). HV antigen was detected in the lungs of one common shrew (*Crocidura russula)* and two red bank voles by ELISA from which no serum was available. No evidence of HV serum antibodies was found in the members of the Muridae, Leporidae, Canidae, Mustelidae, Bovidae, Suidae and Vespertillionidae families (Table 1). No HV antibodies could be demonstrated in the 2025 sera from domestic animals of 14 different species (Table 1). The geographical distribution of all the HV positive animals is shown in Figure 1.

The distribution of the locations where the red bank voles, apparently the main HV resevoir in The Netherlands, were trapped is shown in Table 3. In five of the nine locations where HV seropositive or HV antigen positive animals were detected, the incidence in red bank voles ranged from 25 to 60% per positive area. In four of the five areas where HV infected animals had been identified, clinically and serologically confirmed human cases of NE had been documented.

Epidemiological, clinical and laboratory findings in NE patients

Epidemiological and clinical findings of 27 HV seropositive individuals who had suffered from NE between January 1974 and June 1993 are shown in Figure 2. Symptoms were observed more frequently in males (74%), with a peak between 30 and 40 years in 12/27 (44%) and no cases in children under the age of nine years. HV infections were observed in all seasons with a slight peak in the summer 11/27 (41%). Mapping of the residences of the clinical NE cases showed that 26/27 (96%) of the confirmed cases lived in rural and forested areas located in the eastern and southern regions of The Netherlands (Figure 1).

The most pronounced symptoms in hospitalized patients were: abdominal and/ or flank pain in 24/25 patients, followed by fever (body temperature >38.0 °C) in 25/27 patients, vomiting in 14/25 patients, nausea in 17/25, headache in 12/25 patients and myopia in 4/27 patients. Myopia may have been overlooked in some patients, because it is easily missed during clinical examination. Duration of illness, defined as the first day of illness till discharge from hospital ranged from 11 to 41 days (mean 17 days).

Laboratory findings showed that the serum creatinine was increased (> 110 µmol/l) in all 27 patients, ranging from 140 to 1300 µmol/l with a mean value of 537 µmol/l. Four of the 27 patients had to be given haemodialysis. Thrombocytopenia was found in 10/24 patients. Alanine aminotransferase (ALAT) was increased in 11/27 patients, ranging from 31 to 168 U/l. Protein was detected in the urine of 18/27

patients; in 14/27 the amont of protein was more than 0.5 gr/l and in four of the 27 only a trace protein was detected. Microscopic haematuria, defined as > 5 erythrocytes/µl urine, occured in 14/27 patients. All the data observed coincided well with those published for other NE cases in neighbouring countries [Pilaski et al., 1991; Van Yepersele de Strihou, 1991] as shown in Figure 2.

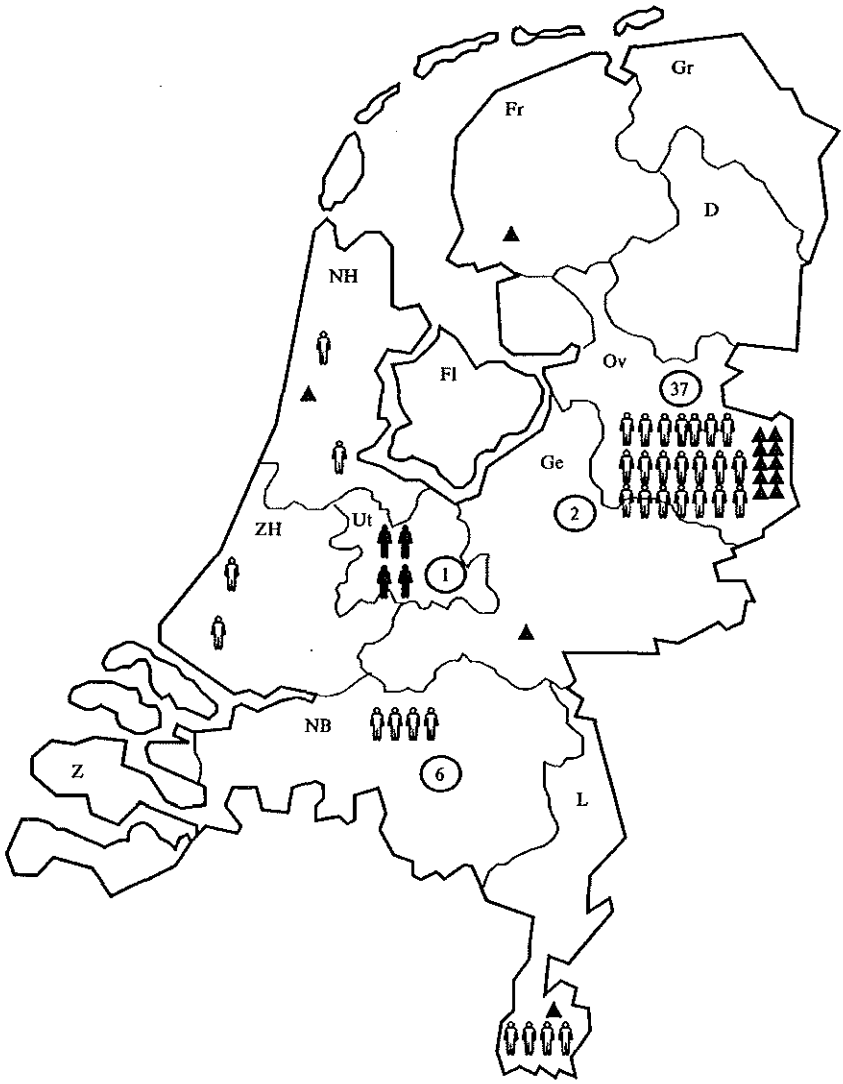

Figure 1. Geographic distribution of hantavirus infections in The Netherlands; ⚕ seropositive laboratory workers; ⚕ .: serologically confirmed human cases of NE; ⊙ : in numbers of seropositive individuals in the province indicated; ▲: HV sero- or antigen positive rodents. (Letter): indicating the different provinces; (Fr): Friesland; (Gr): Groningen; (D); Drenthe; (Ov): Overijssel; (Ge): Gelderland; (Ut): Utrecht; (Fl): Flevoland; (NH): Noord-Holland; (ZH): Zuid-Holland; (Z): Zeeland; (NB): Noord-Brabant; (L): Limburg.

Table 3. Prevalence of HV infections in red bank voles (*Clethrionomys glareolus*) in The Netherlands at different locations.

Location	Year	positive mice/ numbers tested	Percentage %	Reported human NE case
Arnhem (Ge)	84	1/4	25	no
Loenermark (Ge)	86	0/5	0	no
Kempen (B)	86-87	0/22	0	no
Den Bosch (B)	88	0/1	0	yes
Linschoten (Ut)	89	0/11	0	no
Geulen (L)	89	0/21	0	no
Velsen (NH)	89	1/4	25	no
Volthe (Ov)	89	3/5	60	yes
Lutte (Ov)	89	1/4	25	yes
Doldersum (D)	93	0/12	0	no
Bergen (NH)	93	0/4	0	no
Rossum (Ov)	93	3/12	25	yes
Boekelo (Ov)	93	3/6	50	yes
Total	84 - 93	12/111	11	5/9

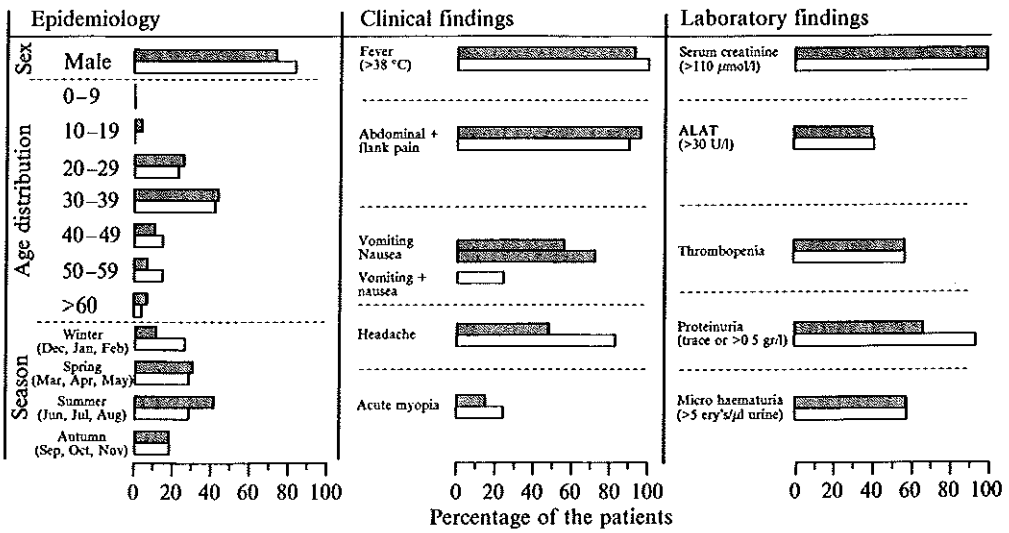

Figure 2. Epidemiological, clinical and laboratory findings of hantavirus nephropathy patients in The Netherlands (▩) Germany [18], Belgium and northern France [19] (□).

Discussion

Here we have documented that HV infections caused by a Puumula-like virus occur in eastern and southern rural and forested areas in The Netherlands, in addition to laboratory-associated HV infections caused by a Seoul-like virus [Groen et al., 1991a, 1991b]. As in neighbouring countries Germany and Belgium, the main reservoir of this infection is the red bank vole (*Clethrionomys glareolus*), which in a limited survey in the apparently enzootic areas showed a seroprevalence of HV-specific antibodies of 25-60%. Evidence of sporadic infection was also found in other animal species: common shrew (*Crocidura russula)* in an apparently enzootic area and in the common vole (*Microtus arvalis).* No evidence for HV infection was found in other feral mammalian species investigated. These included bats, which have recently been shown to be a reservoir for HV infection in Korea [Kim et al., 1994]. Screening of the most common domestic mammalian species in The Netherlands indicated that domestic animals including the cat do not play an important role in the transmission of HV disease to humans. In the United Kingdom, however, cats have been shown to have a relatively high prevalence of HV-specific serum antibodies [Bennet et al., 1990].

Evidence for HV infection in humans was predominantly found in apparently enzootic areas in individuals with typical signs of NE. In contrast to reports from other countries which demonstrated serological evidence of HV infections in individuals with chronic renal disease [Yanagihara, 1990; Glass et al., 1990, 1993; Nuti et al., 1993; Tsianos et al., 1993], we only found evidence for HV infection in one out of 642 haemodialysis and renal transplant patients. Furthermore, the seroprevalence of HV infections in patients suspected of leptospirosis (1%) was slightly lower than the incidence of acute leptospirosis in this group (3%), which indicates the relative importance of the HV infection in The Netherlands. HV seropositive individuals were more frequently identified amongst laboratory workers (2%), foresters (4%) and animal trappers (6%) than in the population at large (<0.5%), which indicates that the majority of these individuals run an occupational risk for acquisition of HV infections. It is of interest to note that the majority of the seropositive individuals identified in these studies had no history of clinically apparent renal disease, indicating that subclinical or non-typical HV infection may occur. The clinical signs of the serologically-confirmed human NE cases in The Netherlands are quite similar to those observed in neighbouring countries, with abdominal or flank pain, elevated levels of serum creatinine and proteinuria as most prominent features. Although most of the non-laboratory associated HV infections run a relatively mild course, more severe cases have been diagnosed. Recently, we also investigated a patient with Guillain-Barré syndrome in association with HV infection [Esselink et al., 1994]. The predominance of cases in males between 30 and 40 years of age, living in rural or forested areas is also quite characteristic for NE cases observed in Western Europe

[Osterhaus et al., 1989; Pilaski et al., 1991; Jordans et al., 1991; Nuti et al., 1993; Clement et al., 1993]. As in Germany, Belgium and France, NE cases occur throughout the year with a slight increase in the summer months. This is probably due to the mild climate, which largely allows rodents to survive outside the direct human environment. This is in contrast to the situation in Scandinavian countries, where rodents tend to seek human shelters in the autumn and winter [LeDuc, 1987]. Consequently, the disease incidence peaks in these seasons. It is difficult to determine whether the recent increase in diagnosed cases of NE in The Netherlands is due to an increased awareness of the disease amongst the medical profession, or to an increased exposure of humans to the infected rodents [Clement et al., 1993; Gerding et al., 1993; Childs and Rollin, 1994]. The recent outbreak of a previously unrecognized HV disease in the USA with a case fatality rate exceeding 60%, probably due to an increased exposure of humans to infected deer mice [Childs and Rollin, 1994], stresses the need for a monitoring system for HV infections in humans and potential host animals.

Acknowledgements

We kindly acknowledge Dr J. Vos, Dr. J. Blankenstein, Dr. A. Moll van Charante, Dr. R. Diepersloot and Dr. W. Wertheim for providing human serum samples and medical data, Dr. R. Herbes, Dr. A.E.J.M. van den Boogaard, Dr. J.T. Lumey, Dr. J v. Oirschot, Mr. T. Brink Mr. A. Lefévre, and Mr. R. van Apeldoorn for providing animal specimens, Mr. H. Broeders, Mr. B.v. Ordel, Ms. L. van Raay, Drs. M. Schokker and Dr. H. Yuping for techical assistance. This work was supported by a grant from the Veterinary Public Health Inspectorate.

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CHAPTER 1 2 3 4 5

Non-human primate model

A macaque model for hantavirus
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Journal of Infectious Diseases 1995 172:38-44

A macaque model for hantavirus infection

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Journal of Infectious Diseases 1995, 172:38-44.

Summary

Cynomolgus macaques (*Macaca fascicularis*) were experimentally infected with Puumala virus (strain Hällnäs), which causes nephropathia epidemica in humans in Western Europe. During the first week after intratracheal inoculation, the monkeys exhibited signs of lethargy followed by mild proteinuria and microhaematuria. Histopathologic changes during the first seven weeks after infection, were largely confined to abnormalities in medullary tubular cells of the kidneys, which coincided with the demonstration of viral antigen and viral RNA. The development of different classes of virus-specific plasma antibodies to the respective viral antigens were similar to those observed in humans with nephropathia epidemica. This first description of a non-human primate model for a hantavirus infection shows that the cynomolgus macaque provides a suitable model to study the pathogenesis of Puumala virus infections and to evaluate new diagnostic methods, immunization strategies and therapies.

Introduction

Nephropathia epidemica (NE) is a form of haemorrhagic fever with renal syndrome (HFRS) in humans, which occurs in Europe and is caused by infection with Puumala virus (PUUV) belonging to the genus *Hantavirus* of the *Bunyaviridae* family. Several hantavirus subtypes have been described [Sugiyama et al., 1987; Lee et al., 1985], including Hantaan-like, Seoul-like, and PUUV-like viruses. Recently, another subtype, named Muerto Canyon virus, which was shown to cause hantavirus pulmonary syndrome in humans in the United States [Nichol et al., 1993]. The spectrum of clinical symptoms caused by infections with the different hantavirus subtypes varies from severe to mild, depending on the viral subtype involved. PUUV infections in humans are usually accompanied by an influenza-like illness at the onset, followed by a transient acute renal failure. Since many infections run a mild course, the true incidence of NE is underestimated in most European countries.

Fewer than 1% of the infections with PUUV-like viruses are fatal. This contrasts sharply with Hantaan-like virus and Muerto Canyon virus infections, which are fatal in about 10% and 60% of cases, respectively [Childs et al., 1994].

The primary pathophysiological findings of HFRS caused by hantavirus infection are largely the result of an increased vascular permeability, and *in vitro* viral replication has been demonstrated in human vascular endothelial cells [Pensiero et al., 1992]. Histopathologic abnormalities have been demonstrated in tubular epithelium cells of hantavirus antigen-positive kidneys. In general, histologic lesions in kidney biopsies of NE patients vary according to the stage of the disease and are usually relatively mild and unspecific [Mustonen et al., 1994]. The most common histopathologic changes in the kidney are flattened tubular epithelium and dilated lumina and occasionally filled with desquamated cells [Collan et al., 1991; Mustonen et al., 1994]. In addition to direct damage due to cytopathic changes in vascular endothelial cells and kidneys, immune-mediated mechanisms are implicated in the pathogenesis of hantavirus infections [Penttinen et al., 1981; Cosgriff et al., 1991].

The different hantavirus subtypes have different reservoir rodent host species, from which they are transmitted to humans, predominantly via aerosols. In the rodent reservoir species, hantaviruses cause persistent and usually subclinical infections [Tsai, 1987]. The main reservoir host species of PUUV the red bank vole (*Clethrionomys glareolus*), becomes infected persistently and subclinically. Because of the apparently different pathogeneses of hantavirus infections in rodents and humans, rodent models are considered of limited value for studying pathogenesis of human hantavirus infections. However, for practical reasons predominantly hantavirus rodent models have been explored [McKee et al., 1985; Tamura et al., 1989; Zhang et al., 1989]. Although non-human primates may develop hantavirus infection, similar to that in humans, little or no attention has been paid to the use of non-human primates for this purpose.

Here we describe the experimental infection of cynomolgus macaques (*Macaca fascicularis*) with PUUV, which results in a mild form of acute renal infection similar to mild forms of NE in humans. This monkey model will allow in-depth studies of the pathogenesis of primate hantavirus infections and the evaluation of new diagnostic methods, immunization strategies, and therapeutic approaches.

Materials and methods

Virus preparation

PUUV strain Hällnäs B1 was passaged in Vero E6 cells in 150 cm^2 bottles using

standard techniques [Groen et al., 1991]. Fourteen days after infection of the cells with an M.O.I. of approximately 10^3 $TCID_{50}$/mL, they were washed with PBS and harvested by three cycles of freezing and thawing. Aliquots of 1 mL of the infected cell lysate were stored at -70°C. To determine virus infectivity titers, ten-fold dilutions of this preparation were made in cell culture medium and mixed with 4 x 10^5 Vero E6 cells in 0.5 mL. After incubation for 1 hour at 37°C the cells were washed and cultured in slide flasks (Nunc, Roskilde, Denmark) for eight days at 37°C. Infected cell cultures were identified by an indirect fluorescence assay (IFA) with a specific rabbit anti-PUUV antibody as described [Groen et al., 1991]. Infectivity titers were calculated and expressed as $TCID_{50}$.

Infection of cynomolgus macaques with PUUV

Cynomolgus macaques were kept in pressurized biological safety glove boxes. Animals (one male, seven female; age range, 2 - 10 years; weight, 2.5 - 7.0 kg) were inoculated intratracheally with 10^6 $TCID_{50}$ of PUUV in 5 mL of PBS by use of flexible canula. After inoculation, monkeys were monitored daily for behavioral changes and clinical signs. Before manipulations, the monkeys were anaesthetized intramuscularly with 10 mg/kg ketamine hydrochloride, and heparinized blood samples were collected for virus isolation, serology, blood chemistry, and hybridisation analysis. In the first experiment two monkeys (7 and 8) were infected, and the various laboratory parameters (serology, serum creatinine and virus isolation) were monitored at various time points (Figure 1), before they were euthanised at week 17 and 30, respectively. In a second experiment the six other animals were euthanatised at predetermined time points. Euthanatised animals underwent complete necropsy; blood samples were collected for the various laboratory parameters and tissues were processed for histopathologic examination. For practical reasons, urine samples could not be collected from the animals; limited amounts of urine could be collected by needle aspiration from urinary bladders of four monkeys after euthanasia.

Clinical chemistry

Immediately after collection, sodium citrate blood samples were sent to our clinical laboratory, where blood was analyzed for potassium, albumin, liver enzyme alanine aminotransferase (ALAT) and serum creatinine levels by use of an autoanalyzer. Interleukin-2 was measured in plasma of the respective monkeys with a commercially available ELISA kit (CLB, Amsterdam, The Netherlands). Urine sediment was immediately measured after urine was collected. Proteinuria was measured by urine dipsticks.

Histochemistry and immunostaining

For the histopathologic examination of the organs, specimens were fixed in 4% buffered formaldehyde, paraffin-embedded, cut in sections 4 - 5 µm thick, stained with haematoxylin-eosin and scored: -, no abnormalities; +, histopathologic abnormalities.

Immunohistochemical staining using a three step method, was done with monoclonal antibodies directed against group-specific epitopes of the viral glycoprotein G2 (8E10; 11E10; 3D7) and the nucleoprotein (GB04, AA07) [Arikawa et al., 1989; Ruo et al., 1991]. All were of the IgG1 isotype. The specificities of the glycoprotein-specific monoclonal antibodies in immunostaining have been demonstrated [Kim et al., 1993]. Monoclonal antibodies to rabies virus glycoprotein of the same isotype (2-22C5) was used as control. Paraffin sections for immunostaining were mounted on 3-aminopropyl-3-etoxisilane-coated slides, cleared, and endogenous peroxidase activity was blocked in methanol with H_2O_2 (0.3% vol/vol). They were then washed in distilled water and processed for immunostaining. Monoclonal antibodies and conjugates were titrated for the optimal working dilution in PBS supplemented with bovine serum albumine (1% wt/vol), normal monkey serum (2% vol/vol) and normal swine serum (2% vol/vol). Slides were incubated with the monoclonal antibodies for 60 min at room temperature, washed three times for 10 min with PBS + Tween 20 (0.5% vol/vol), incubated with rabbit anti-mouse IgG horseradish peroxidase conjugate (DAKO, Glostrup, Denmark) and swine anti-rabbit IgG horseradish peroxidase conjugate (DAKO), respectively. Binding of the respective antibodies to the antigens was visualised with a Nickel-enhanced diaminobenzidine peroxidase reaction to obtain black deposits. For the positive and negative controls, Vero E6 infected with PUUV and uninfected cell pellets were embedded in paraffin and processed in the same way as the monkey tissues.

Immunoperoxidase staining was scored on an arbitrary scale: -, negative; +, positive; ++, strong positive.

Virus isolation

For the first experiment heparinized peripheral blood was separated into plasma and peripheral blood mononuclear cells (PBMC) by discontinuous gradient centrifugation on a dextran-500 (4% wt/vol)/sodium-metrizoate (32% wt/vol) solution. PBMC were washed twice and suspended in medium containing dimethyl sulfoxide (10% vol/vol) plus fetal calf serum (10% vol/vol) at a density of 10^6 cells/mL and stored at -135°C until all samples were collected. Plasma samples were divided into 200 µL portions and stored at -70°C. PBMC (10^6) and 200 µL plasma were cultivated with Vero E6 cells. After three passages the monolayers were

washed with PBS and the cells were fixed with ethanol at -70°C.

From all the monkeys, kidney organ suspensions were made in medium containing fetal calf serum (10% vol/vol) using glass grinders and these suspensions were used for virus isolation in Vero E6, primary cynomolgus macaque kidney cells, and human embryo kidney and lung cells using the same procedures.

The presence of viral antigen was shown according to the method for virus preparation described above.

Serological assays

PUUV-specific antibodies were measured in all the plasma samples collected. Before use, the plasma was heat inactivated for 30 min at 56°C. In the first experiment, IgM-specific plasma antibodies were measured with an IgM-capturing ELISA as described previously for human hantavirus-specific IgM antibodies, using cross-reactive IgM-specific antibodies [Groen et al., 1992]. Specific IgM plasma levels were expressed as the differences between the A_{450} obtained with PUUV antigen and the A_{450} obtained with Vero E6 control antigen (Figure 1). In the second, experiment the hantavirus-specific IgM antibodies were analyzed in a commercial available IgM capture ELISA (Progen, Heidelberg, Germany) and expressed according to the kit instructions, as one (+) and two times (++) above the cut-off value.

For the detection of PUUV-specific IgA and IgG antibodies an IFA was used, essentially as described for detection of human PUUV-specific immunoglobulin [Groen et al., 1991,

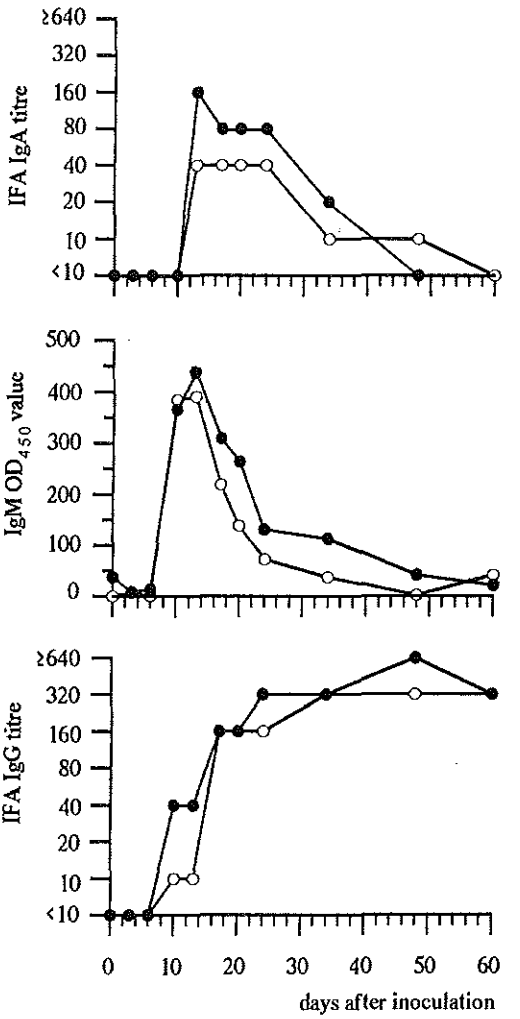

Figure 1. Development of Puumalavirus specific plasma IgA, IgM and IgG antibodies in 2 monkeys infected intratracheally. Open and closed symbols represent monkeys number 7 and 8, respectively.

1992]. Briefly, monkey IgA specific for PUUV was detected with a rabbit anti-monkey IgA fluorescein isothiocyanate conjugate (Nordic Laboratories, Tilburg, Netherlands). Monkey IgG antibodies were detected using a specific goat anti-human IgG fluorescein isothiocyanate conjugate (Cappel, Organon Technica, Turnhout, Belgium). Titers determined in both IFAs were expressed as the reciprocal of the antibody dilution still giving a clear characteristic dot-like immunofluorescence pattern. For measurement of the antibody response against the structural proteins of PUUV, plasma samples were tested in a recently described inhibition ELISA [Groen et al., 1992]. The IgG response against the viral nucleoprotein was also analyzed in a commercial available IgG ELISA (Progen) and expressed in units/mL according to the kit instructions.

RNA extraction and hybridisation

Kidney tissue for hybridisation was stored at -80°C until analysis. Total RNA was extracted from kidney tissue by a modification of the guanidinium method. Kidney tissue (approx. 0.5 g [wt/vol]) was homogenized in Eppendorf cups with 500 µL solution D (4 M guanidine thiocyanate, 25 mM sodium-citrate [pH 7.0], sarosyl (0,5% wt/vol), 100 mM beta-mercaptoethanol and 20 µg poly A/mL) using a disposable grinder. PUUV-infected and uninfected cell pellets were treated the same way and used as positive and negative controls. The homogenate was extracted with phenol-chloroform-isoamylalcohol [25:24:1] and chloroform-isoamylalcohol [24:1], respectively. Total RNA was precipitated with 600 µL 2-propanol and 35 µL 3 M sodium acetate (pH 5.2) at -20°C for 18 hours. Precipitates were pelleted at 10.000g at 4°C and washed once with 500 µL cold ethanol (80% vol/vol). Air-dried pellets were resuspended in 50 µL diethylpolycarbonate treated-H_2O. Total RNA (5 µg) was spotted on Hybond N+ membranes (Amersham, UK) and cross-linked by UV irradiation for 2 min. Hybridisation done out with ^{32}P labeled M (AATTTAAATGCAGGTTGGACAG) - and S (GCACCAGATCGGTGCCCCCC) [Giebel et al., 1989; Stohwasser et al., 1990] -specific oligonucleotide probes. The intensity of the hybridisation signals was scored on an arbitrary scale: -, negative; (+), weak; +, positive; ++, strong positive.

Results

Development of clinical signs

After infection, all eight monkeys lost their apatite and became clearly apathetic from day two to six after infection. Four developed skin rash between day 9 and 11,

Table 1. Results of clinical chemistry, serology, histopathology, antigen and viral RNA detection in experimentally infected monkeys with PUUV at different times after inoculation.

Parameters	Monkey number and time of euthanasia after inoculation							
	1 6 days	2 11 days	3 2 weeks	4 3 weeks	5 7 weeks	6 10 weeks	7 17 weeks	8 30 weeks
Clinical chemistry								
ALAT (number of equivalents of pre-infection value)	1	1	8	1	1	1	1	1
IL-2 (number of equivalents of pre-infection value)	6	11	1	1.5	1	1	NT	NT
Proteinuria (g/l)	neg	neg	1.0	0.3				
Haematuria (RBC/µL)	neg	10	50-150	neg				
Serology								
IgM (ELISA cut-off value)	-	++	++	-	+	-	-	-
IgA (IFA titer)	<10	40	320	<10	80	80	<10	<10
IgG (IFA titer)	<10	<10	10	<10	320	320	160	320
IgG NP (ELISA units/mL)	<25	<25	<25	<25	66	70	27	47
NP (inhibition ELISA %)	<10	<10	<10	<10	62	47	76	73
G1 (inhibition ELISA %)	<10	<10	<10	<10	53	10	<10	31
G2 (inhibition ELISA %)	<10	<10	<10	<10	20	23	<10	60
Histopathology, Ag and RNA detection								
Histopathological changes*	+	+	+	+	+	-	-	-
Antigen detection* : G2 (immunoperoxidase)	-	+	++	++	+	+	-	-
: NP (immunoperoxidase)	-	+	++	+	+	-	-	-
Dot-spot hybridisation*	(+)	++	++	+	(+)	(+)	NT	(+)

Foodnote
PUUV: puumalavirus
ALT: alanine aminotranferase
IL-2: interleukin-2
RBC: red blood cells
NP: nucleoprotein
G1 or G2: glycoprotein 1 or 2
* Arbitrary scored (see M and M)
NT: not tested

which was most prominent at non-pigmented skin areas like their faces. During the remaining period, no clinical signs were observed. Serum chemistry analyses for all monkeys yielded normal values throughout the study, including serum potassium, serum creatinine and albumin levels. Plasma ALAT levels were measured in monkeys 1-5 at the day of euthanasia, and an increase was only found only in monkey 3 (Table 1). Plasma interleukine-2 levels at the time of euthanasia, were increased in monkeys 1 and 2 (euthanatised at day 6 and 11, respectively) and not in monkeys 3-8 (euthanised at week 2, 3, 7, 10, 17 and 30, respectively) (Table 1). For the four monkeys from which urine could be collected on the day of euthanasia, urinalysis was done. Slight proteinuria was seen in monkey 3 (euthanised at 2 weeks) and monkey 4 (euthanatised at 3 weeks) with protein levels of 1.0 and 0.3 g/l, respectively.

Microhaematuria was demonstrated in monkeys 2 (euthanised at 11 days) and 3 (euthanatised at 2 weeks), with 5-10 and 50-150 red blood cells/μL, respectively (Table 1).

Pathologic findings

Gross postmortem examination on the day of euthanasia demonstrated no abnormalities in brains, aorta, kidneys, liver, spleen, heart, lung, urinary bladder and multiple locations in the gastrointestinal tract. Histopathologic examination of these organs, showed only abnormalities in the tubules of the kidneys, limited to the medullary epithelium and consisted of pycnotic nuclei and desquamated cells in monkeys 1-5 at the time of euthanasia (Table 1). In monkey 3, euthanatised at 2 weeks, desquamated epithelial cells present in the tubuli were most prominent. The tissues of monkeys 6-8 (euthanatised at week 10, 17 and 30, respectively) showed no lesions related to the infection. No histologic changes in glomeruli and cortex histology were observed in any of the monkeys.

Virus isolation

Virus isolation procedures with all the plasma and PBMC samples collected at various time points (as seen in Figure 1) from monkeys 7 and 8 in the first experiment and with kidney suspensions from all the monkeys, were unsuccessful when three subsequent passages were done. Cell substrates used in these experiments included Vero E6, primary cynomolgus macaque kidney, and human embryo kidney and lung cells, which all were permissive to infection with the stock PUUV used for the intratracheal inoculation in the first passage (not shown).

Immunoperoxidase staining for viral proteins in monkey kidneys

By immunoperoxidase techniques PUUV-specific glyco- and nucleoproteins were

detected in kidney sections collected from day 11 to week 10 after infection. Both viral antigens were located in the cytoplasm of renal tubular epithelial cells in the cortical and medullary areas of the kidneys (Figure 2a,b, Table 1). Two weeks after infection the presence of viral antigens in the tubuli was most pronounced. The distribution of the antigen positive tubules was largely focal and mainly located in the area between cortex and medulla. No non-specific staining was observed with isotype matched control antibodies.

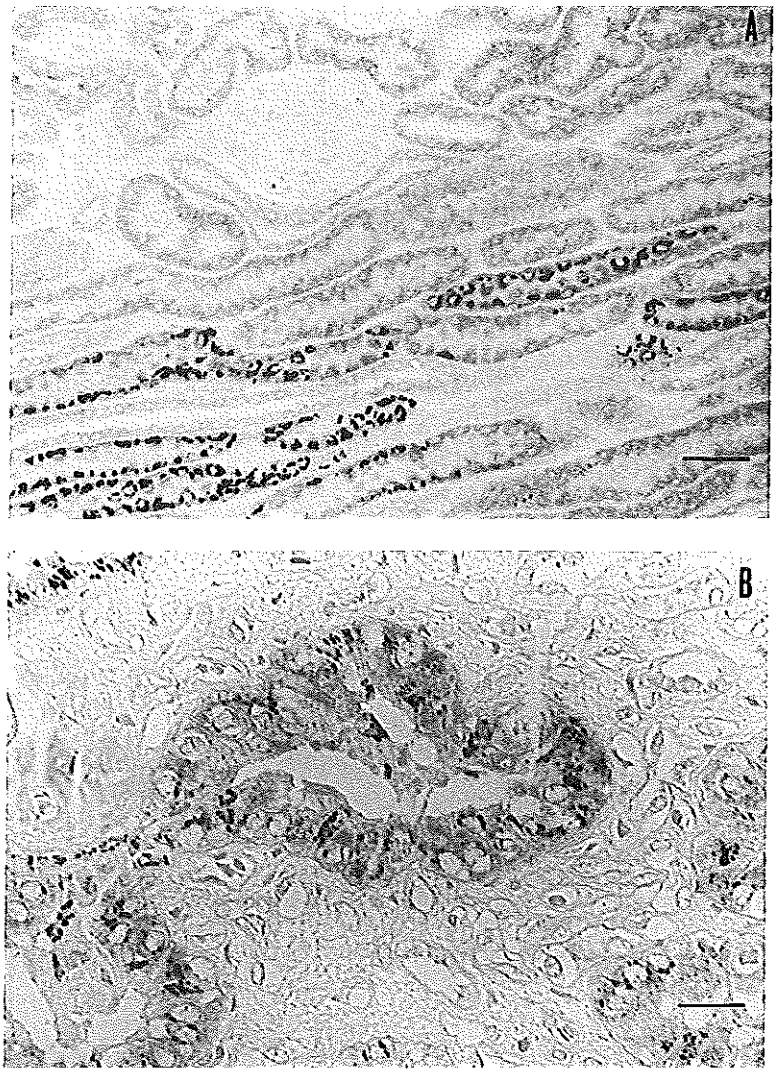

Figure 2. Puumala virus-specific positive immunoperoxidase staining in kidney tissue from monkey no. 3. **A**, Epithelium of straight tubules of cortical area; convoluted tubules and glomerulus are negative (bar = 100 μm). **B**, Collecting tubule of medulla (bar = 25 μm).

Development of PUUV- specific IgM, IgA and IgG plasma antibodies

In the first experiment, both animals (7 and 8) infected with PUUV were tested for the development of IgM, IgA, and IgG specific plasma antibodies at regular intervals (Figure 1). The peak of PUUV specific IgM antibodies was found at day 13 in both animals and reached background levels at day 34 and 48, respectively. The peak of IgA antibody titers was at day 13 in both animals and started declining at day 24 to disappear at day 48 and 60, respectively. IgG antibodies were not detected before day 10 and reached a plateau between days 24 and 60.

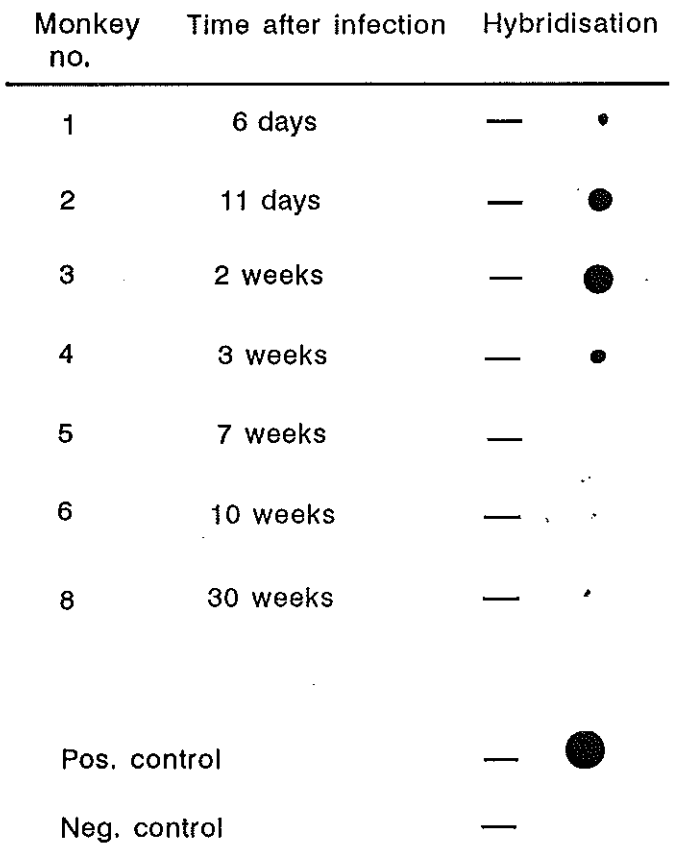

Figure 3. Dot-spot hybridisation signal of PUUV specific viral RNA extracted from kidney cells of experimentally infected monkeys with PUUV at different times after inoculation.

In the second experiment, the six other monkeys were tested for PUUV-specific antibody development at the day of euthanasia. In monkey 4 (euthanatised at 3 weeks), no PUUV-specific plasma antibodies were found. In the other five monkeys, the presence of PUUV-specific IgA, IgM and IgG plasma antibodies, coincided with the patterns found in the two monkeys of the first experiment (Figure 1, Table 1). IgG plasma antibody levels measured with a commercially available ELISA against an *Escherichia coli* expression product of the nucleoprotein of PUUV, were <25 units/mL in monkeys 1-4, were relatively high in monkeys 5 and 6 (plasma antibody levels of 66 - 70 units/mL), and were slightly lower in monkeys 7 and 8 (plasma antibody levels of 27 - 47 units/mL) (Table 1). Antibodies against the viral glycoproteins G1 and G2 and the nucleoprotein were not detected with the inhibition ELISA in monkeys 1-3. Plasma antibodies against the G1 and G2 were detected in the plasma of monkeys 5, 6 and 8 in the inhibition ELISA with inhibition levels of 10% - 53%, but not in monkey 7 (Table 1). In the plasma from monkeys 5-8, antibodies against the nucleoprotein were detected in the inhibition ELISA with inhibition levels of 62% - 73%.

Determination of virus-specific RNA

The hybridisation assay with RNA extracted from kidney cells collected at different intervals after initial infection using oligonucleotide specific probes for the M-RNA and S-RNA segment are shown in table 1 and figure 3. Positive results were obtained with samples from all the tested monkeys; monkey 7 which could not be tested for logistical reasons. The highest amounts of viral RNA were detected in monkeys 2 and 3, euthanatised at day 11 and week 2, respectively (Table 1, Figure 3).

Discussion

In the present, study we have shown that the cynomolgus macaque is susceptible to intratracheal infection with PUUV, which apparently causes a mild NE without clear functional abnormalities. We used the intratracheal route in these experiments, since the most common mode of hantavirus infections in humans is probably through aerosols via the respiratory tract [Tsai, 1987]. The clinical symptoms observed, were similar to those found in humans with NE, with a transient proteinuria. The skin rash was not observed in all the infected monkeys, possibly because of pigmentation of the skin. A rise in plasma interleukine-2 levels is often also observed in humans with hantavirus infections in concordance with the degree of illness [Huang et al., 1994]. Other symptoms, such as general malaise and apathy, flank pain or fever, are more

difficult to assess in monkeys. Changes in blood chemistry values were not found, except for a rise of the liver enzyme ALAT in monkey 3 which did not show histopathologic abnormalities of the liver when it was euthanatised 2 weeks after inoculation. The anaesthesia and changing housing conditions may have influenced these parameters [Verlangieri et al., 1985].

Epidemiologic studies in areas in which PUUV is endemic have shown that the prevalence of hantavirus specific serum antibodies is much higher than the actual numbers of individuals with a history of NE would suggest [Clement and Van der Groen, 1987; Niklasson, 1987; Groen et al., 1995]. This indicates that also in humans, PUUV infections usually run a milder course than the severe cases of NE which require hospitalization. Thus, the observed clinical signs of PUUV infection in cynomolgus macaques may be quite similar to the relatively mild symptoms usually observed in humans. With the exception of medullar interstitial haemorrhages noticed in approximately 60% of the kidney biopsies of NE patients, histopathologic abnormalities in the tubules are usually mild and unspecific [Mustonen et al., 1994]. Histopathologic changes in the monkeys, appeared to be related to transient abnormalities in the renal tubular epithelium, correlate with the findings in humans with NE [Collan et al., 1991; Mustonen et al., 1994]. The histopathologic lesions in the monkeys, clearly coincided with the presence of viral antigen as well as RNA in the kidneys (Table 1). The distribution of viral antigen in the kidneys was focal and restricted to the tubular epithelium. This finding together with the typical intracellular localization of the antigen, resulted in a pattern similar to that observed in humans with HFRS [Kim et al., 1993]. This suggests that during a period of approximately 7 weeks after infection, virus replication takes place in the kidneys. Direct involvement of tubular epithelial cells was also suggested by others studying kidney biopsies of patients with Hantaan virus infections [Collan et al., 1991; Kim et al., 1993]. The virtual absence of infiltration of lymphoid cells or any other signs of inflammatory reactions at this site during this period would be unexpected but has also been documented in humans with NE [Van Ypersele De Strihou and Mery, 1989; Collan et al., 1991]. This indicates that the direct effects of infection of tubular epithelium, rather than a local inflammatory reaction, are the primary cause of the acute renal symptoms. It is puzzling why isolation of the virus from the kidneys of these animals during these experiments remained unsuccessful after three passages, although the cell substrates used were susceptible to infection with the PUUV preparation used for infection of monkeys (not shown). Others have shown that after nine passages of human specimens on Vero E6 cells, no PUUV-specific antigen could be detected by indirect IFA [Grankvist et al., 1992]. These and our findings indicate the difficulties to isolate PUUV from human and primate specimens, which contrasts sharply with the relative ease with which

Hantaan virus can be isolated from individuals with HFRS [Qiang et al., 1989]. This may indicate that little infectious virus is formed in the kidneys or that the presence of virus neutralizing antibodies interferes with replication of the virus *in vitro*.

The outline of the experiment, in which PUUV-infected monkeys were subjected to regular blood sampling or were euthanatised at regular intervals, allowed us to study the kinetics of several parameters, such as the presence of histologic changes, viral antigen, RNA and antibody development. The kinetics of virus specific IgM, IgA and IgG plasma antibodies coincided well with previous observations in humans at different stages of the infection [Groen et al., 1992, 1994]. The kinetics of the plasma antibody responses of the monkeys against the individual structural proteins is also largely similar to in humans with a PUUV-like infection [Groen et al., 1992]. The kinetics of the antibody responses against the nucleoprotein and the G2 proteins were almost identical to those observed in humans. Antibody responses to G1 protein, which are quite marked in the acute phase of the infection in humans, appeared to be rather low in the infected monkeys studied (Table 1). In monkey 4, which was euthanatised 3 weeks after infection, no virus-specific plasma antibodies were detected, although viral antigen and RNA were demonstrated in kidneys. This shows that like in humans [Pether et al., 1993] some individual animals may have very poor or slow antibody responses, complicating the diagnosis of the infection, which at present is almost exclusively based on serology.

Taken together our data show that PUUV infection of cynomolgus macaques is a suitable model to study the pathogenesis of NE in humans. For practical reasons, studies on the pathogenesis of the early stages of the infection, in which the most alarming clinical phenomena are found, have been limited in humans. This monkey model may be valuable for studying the changes that lead to the acute symptoms of anuria followed by polyuria early after infection with PUUV. Monkey infection studies with other hantavirus subtypes, causing more severe renal or respiratory disease in humans, may also prove to be valuable to understanding the pathogenesis and variability of their clinical presentations. Finally, the model described will allow the evaluation of new diagnostic methods and of the efficacy and safety of candidate vaccines and therapies for PUUV and possibly other hantavirus infections.

Acknowledgements

We thank Dr. A. Sanchez (CDC, Atlanta, USA), the late Dr. J.M. Dalrymple (USAMRIID, Frederick, USA) for providing the monoclonal antibodies; Joke Robinson, Nico Schmidt (RIVM, Bilthoven, The Netherlands), and Edwin Fries for technical assistance; Rob van Binnendijk (Erasmus University, Rotterdam) and

Guido van der Groen (Institute of Tropical Medicine, Antwerp, Belgium) for helpful suggestions; and Conny Kruyssen for handling the manuscript.

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CHAPTER 1 2 3 4 5

Summarizing discussion

The present thesis describes the development of methods for the diagnosis of human and animal hantavirus infections and the use of these methods to study the clinical course and epidemiology of these infections in The Netherlands. In addition a non-human primate model is described which will allow in depth studies of the pathogenesis of primate hantavirus infections and the rational evaluation of diagnostic methods and future vaccines for hantavirus infections.

Hantavirus infections causing HFRS first drew major attention of western physicians during the Korean war when many United Nations sanctioned soldiers suffered from a serious disease which caused many deaths amongst them [Earle, 1954]. About 25 years later, Lee and co-workers associated the occurrence of this disease with the presence of rodents in endemic areas. They isolated the causative virus from a Korean striped field mouse (*Apodemus agrarius*), and named it Hantaan virus after the Hantaan river in Korea. This virus had not been isolated previously from humans or animals and was characterized as a new member of the *Bunyaviridae* family. A separate genus was established within this family - the genus *Hantavirus* - of which Hantaan virus became the prototype virus. Sofar this proved to be the only genus of the *Bunyaviridae* family, of which the members are not transmitted by arthropods, but by direct or indirect contacts with secreta or excreta of infected rodents.

The presence of hantavirus infections in The Netherlands was first identified in 1984 when it was shown that laboratory workers of the National Institute of Public Health and Environmental Protection in Bilthoven had been infected with a Hantaan-like virus. They had been infected by contacts with infected laboratory rats imported from Belgium [Osterhaus et al., 1984] (chapter 3). Two of these laboratory workers had developed serious renal disease, that could retrospectively be attributed to this hantavirus infection. The IFA initially used to screen samples from the laboratory animals and humans involved in this outbreak, was based on Hantaan virus (strain 76-118) infected Vero E6 cells. This assay was subsequently used to carry out initial screening programs amongst patients with disease symptoms indicative for leptospirosis in The Netherlands. This study showed that hantavirus infections causing a mild form of HFRS, better known as NE, occur in The Netherlands.

Laboratory diagnosis of hantavirus infections

Patients presenting acute symptoms of a hantavirus infection (chapter 2), are often not recognized as such, on basis of their clinical symptoms alone [Lee and Van der Groen, 1989]. To allow serological diagnosis of acute hantavirus infections, clinicians should be encouraged to collect serum samples from patients who have

been in endemic areas and present these symptoms. For the detection of hantavirus specific antibodies in serum samples an IFA was initially used [Lee and Lee, 1976]. For this purpose lung sections of mice naturally infected with Hantaan-like virus were first made. Subsequently, drop slides prepared with Vero E6 cells infected with Hantaan virus (strain 76-118) [Van der Groen and Beelaert, 1985] were used. The antigen preparations of the hantavirus infected lung sections, but also the drop slides gave "atypical" immunofluorescence patterns in up to 6% of the samples tested (chapter 2). It was not clear initially, whether this aberrant pattern of the IFA was due to infection with other hantavirus subtypes, or to non-specific reactivity of the sera concerned. By western-blot analysis, using Hantaan virus as a reference, it was demonstrated that most of these "atypical" reactions were indeed non-specific (chapter 2).

It was also demonstrated that sera from NE patients from Western European showed higher antibody titers in the IFA with homologous Puumala-like virus isolates than with heterologous Hantaan-like virus isolates. In contrast patients with severe HFRS from Asia showed the opposite seroreactivity pattern in the IFA [Sugiyama et al., 1987; Zöller et al., 1989]. These relative one-way cross reactivities between Hantaan virus on the one hand and Puumala virus on the other were also seen in western-blot analyses, immunoprecipitation and virus neutralization assays [Dantes et al., 1987; Lee et al., 1985; Zöller et al., 1989]. Consequently for the serodiagnosis of HFRS the WHO recommended to use the prototype Hantaan virus as well as Puumala virus antigen preparations [WHO, 1991]. To date, the IFA is still used as the standard assay for the detection of hantavirus specific antibodies in many laboratories. However, the production of IFA slides requires P3 facilities which constitutes a major hindrance for the preparation of diagnostic assays in many diagnostic laboratories.

Serodiagnosis of acute hantavirus infections

In hantavirus endemic areas the seroprevalence of hantavirus specific antibodies amongst humans may range from about 0.5 to 2%. Therefore serological diagnosis of hantavirus infection in patients with acute symptoms in these areas cannot conclusively be made by standard measurements of specific IgG serum antibodies. A number of assays based on different principles has therefore been developed to evaluate the time elapsed after onset of the hantavirus infection, using a single serum sample from patients with acute symptoms. The IFA readily detects hantavirus specific IgA, IgM, and IgG antibodies, of which the first two are virtually only present in the acute stage of the infection (chapter 2). The detection of specific IgM antibodies, by indirect systems like the IFA and the ELISA, may be complicated by the presence of rheumatoid factor or high serum IgG levels. Therefore, we and

others have developed alternative methods to determine whether hantavirus specific IgM or other specific antibody classes and subclasses can be detected in patients with acute symptoms indicative for hantavirus infection (Table 1). IgM capture ELISA's as described in chapter 2 do not suffer from the presence of rheumatoid factor or high serum IgG levels [Niklasson et al., 1990; Zöller et al., 1993]. An alternative, rapid and sensitive method using only a single serum sample which also does not suffer from the problems mentioned above, is the IgG avidity assay (Table 1). It is based on the principle that specific IgG antibodies appeared to have a low avidity in the acute phase and higher avidity in the convalescent phase of the infection. These differences can be determined by measuring the dissociation of the antibody/antigen binding with 8M urea.

Table 1. Assays for the diagnosis of acute hantavirus infections

Diagnostic principle	Assay	Detection	Time needed	References
Serology	IFA (indirect)	IgA, IgM, IgG	1-2 hours	chapter 2
	IFA (avidity)	IgG	1-2 hours	in chapter 1
	CFA	Ig total	2 days	in chapter 1
	HIA	Ig total	3 hours	in chapter 1
	HDPA	Ig total	1-5 minutes	in chapter 1
	ELISA (indirect)	IgM,IgG	3 hours	chapter 2
	Capture ELISA	IgA,IgM, IgG1-4	3-4 hours	chapter 2
	CTB-ELISA	Ig total	2-3 hours	chapter 2
	Cell-ELISA	Ig total	2-3 hours	chapter 2
	Competition ELISA	Ig total	2-3 hours	chapter 2
	PRNT	Ig total	several days	chapter 2
Antigen detection	Capture ELISA	antigen	3-4 hours	chapter 3
	Immunohistochemistry	antigen	4-6 hours	chapter 2
RNA detection	Hybridization	RNA	2 days	chapter 4
	RT-PCR	RNA	2 days	in chapter 1

The demonstration of specific IgG antibodies of different subclasses in an acute phase serum may also be indicative for an ongoing hantavirus infection. Using capture assays we found that in the acute phase of hantavirus infections high levels of specific IgG3 antibodies were present whereas IgG1 antibodies were the predominant subclass in the late convalescent phase (chapter 2). Using *Hantavirus* genus specific monoclonal antibodies directed against epitopes of the structural proteins, we also demonstrated different reactivity patterns at different times after infection. High levels of antibodies directed against G1 glycoprotein and nucleoprotein were predominantly found in the acute phase, whereas G2 glycoprotein reactive antibodies

were more prevalent in the late convalescent phase. The development of hantavirus specific IgG1 antibodies was shown to largely parallel the development of antibodies directed against the glycoprotein G2 (chapter 2). From the data presented in chapter 2 it may be concluded that the combination of these two principles provides a reliable tool for the diagnosis of acute hantavirus infections.

Technical aspects of laboratory assays

Some of the disadvantages of the IFA, like "atypical" staining (see above), interpretation problems and inadaptability to automation may be overcome by ELISA systems recently developed for HFRS diagnosis: In chapter 2 we demonstrated that different ELISA systems were at least as specific and sensitive as the IFA systems which were commonly used at that time. These ELISA systems, based on different principles detect hantavirus specific antibodies with inactivated antigen preparations and may be adapted to use in the screening of large serum panels, thus allowing their incorporation in automated systems. These systems have the additional advantages of objective interpretation and ease of standardization. One of the ELISA developed, the CTB-ELISA which is based on hantavirus specific polyclonal antibodies, can both be used for antibody and antigen detection (Table 1).

Although the IFA is relatively simple to perform in comparison to the ELISA systems, both systems are relatively time consuming. For the rapid diagnosis of hantavirus infections a HDPA has also been developed (Table 1), which proved to show a good correlation with the IFA and the ELISA [Tomiyama and Lee, 1990]. An additional advantage of the HDPA could be that no special equipment or highly qualified personnel is needed and that the results can be read with the naked-eye within one hour.

A general disadvantage of all the above described diagnostic assays is that live virus is used for the production of the respective hantavirus antigens. In recently developed ELISA for the detection of hantavirus specific IgM and IgG antibodies, which are based on μ-capture and indirect assays, baculovirus and E.coli-expressed recombinant Hantaan- and Puumala virus nucleocapsid proteins have been used as antigens [Schmaljohn et al., 1988; Rossi et al., 1990; Zöller et al., 1993].

Since hantaviruses are difficult to isolate, especially from human patients, several assays systems have been developed now, which detect viral proteins or RNA directly in infected tissues and organs (Table 2). In chapter 2 we described the development of a CTB-ELISA for this purpose. This assay can be used for the detection of viral antigen in cell culture and biopsy or autopsy specimens from animals or humans. Using this assay we were e.g. able to demonstrate hantavirus antigen in the lungs of rodents caught in endemic areas in The Netherlands (chapter 3). The detection of hantavirus proteins in HFRS patients is only possible in kidney

biopsy or autopsy materials with immunoperoxidase staining, using monoclonal antibodies directed against cross-reactive epitopes of the nucleoprotein and the glycoproteins. Some of these antibodies also appeared to react with viral antigen in formalin-fixed tissues [Kim et al., 1993; Poljak and Avsic-Zupanc, 1994; Zaki et al., 1995]. Using this technique in a retrospective study, we demonstrated Puumala virus antigen in the tubules of kidney biopsies from Dutch NE patients (chapter 2).

Since the majority of the HFRS patients develop a serum antibody response against the hantavirus involved which can be demonstrated with the above mentioned techniques, confirmation of the clinical and laboratory diagnosis by RT-PCR is of less value. Although the RT-PCR is of less value for routine diagnosis, this technique may be useful in those cases where the antibody response is beyond the dection level of the current serological assays or in those cases in which not previously recognised hantavirus subtypes are involved [Nichol et al., 1993]. For the subtyping of hantaviruses the RT-PCR, using a selected panel of subtype specific primers, proves to be a powerful tool [Giebel et al., 1990; Puthavathana et al., 1992; Plyusnin et al., 1994]. It is interesting to note that the recent outbreak of HPS in the USA could be related to infection with a hantavirus by extensive serological studies followed by RT-PCR assays [Nichol et al., 1993]. Subsequently RT-PCR amplified products from the lungs of deceased HPS patients were expressed in E.coli and used as antigen for serological screening, even before the virus responsible for the HPS outbreak was isolated [Feldmann et al., 1993].

Epidemiological aspects of hantavirus infections in The Netherlands

Several of the serological ELISA systems described in chapter 2, were used to study the prevalence of hantavirus infections in humans and animals in different areas of The Netherlands (chapter 3). The first hantavirus screening programmes conducted in The Netherlands carried out with ELISA systems were based on antigen of the prototype Hantaan virus (strain 76-118). From 1984 onwards, several different hantavirus strains were isolated at different continents, including Seoul-like virus, Puumala-like virus, and Prospect-Hill-like virus. The IFA and ELISA systems described in chapter 2 for the serodiagnosis of Hantaan virus infections were adapted to allow the serodiagnosis of the other hantavirus infections as well. Subtype specific ELISA systems were used to study the prevalence of the respective hantaviruses circulating in humans in The Netherlands (chapter 3). This was considered important, since it had been shown that the clinical symptoms of HFRS at different places in Europe differ and that different subtypes may be involved [Antoniadis et al., 1987; Avsic-Zupanc et al., 1989; Settergren et al., 1989; Van Yepersele De Strihou and

Mery, 1989]. Analysis of post infection sera from patients with a retrospectively confirmed hantavirus infection, demonstrated that the infected laboratory workers of the National Institute of Public Health and Environmental Protection as well as those of an institute in Brussels had the highest reactivity in IFA and ELISA against Hantaan-, and Seoul-like viruses. In contrast, the majority of the individuals with a proven non-laboratory associated infection in The Netherlands and Belgium proved to exhibit the highest serum antibody reactivity in ELISA and IFA against Puumala-like virus (chapter 2). However, two individuals, one in The Netherlands and one in Belgium showed a higher reactivity against Hantaan/Seoul-like virus. These hantavirus infections were non-laboratory associated. Comparative analysis of these two human sera together with a panel of sera from individuals with confirmed hantavirus infections in Slovania, using Puumala-like, Seoul-like and Dobrava virus as antigens, showed that these two individuals had probably been infected with a virus more closely related to Seoul virus than to Puumala virus (chapter 3). The newly identified Dobrava virus [Avsic-Zupanc et al., 1992] proved to be closely related to Seoul-like virus. This is in agreement with recently published nucleotide sequence data [Xiao et al., 1993]. The data also confirm that in Slovania different hantavirus subtypes circulate independently [Avsic-Zupanc et al., 1989] and together with our observations presented in chapter 3, indicate that also in The Netherlands more than one subtype may be present in feral rodents. Similar findings were described by Pilaski and co-workers in an area in Germany adjacent to the hantavirus endemic area in The Netherlands [Pilaski et al., 1991]. To study the geographical distribution of hantavirus infections, and to identify the reservoir host species in The Netherlands, serum samples from humans with and without renal disease or with suspected occupational risk, and from feral and domestic animals were collected at several locations between 1984 and 1994. These samples were tested for the presence of hantavirus specific antibodies. In addition, clinical and laboratory findings of individuals with a history of serologically confirmed NE infections were collected. It was demonstrated that the majority of the clinical and sub-clinical human cases of hantavirus infections occurs in rural and forested areas in the eastern and southern parts of The Netherlands. Interestingly, the areas involved proved to form a continuum with hantavirus endemic areas across the eastern and southern borders of the country, in Germany and Belgium, respectively. Human cases of NE in The Netherlands and neighbouring countries were largely confined to those areas where also hantavirus infected rodents were found. In an extensive screening program of several hundreds of patients with renal disease in The Netherlands for the presence of hantavirus infections, it was shown that besides patients suspected of NE, predominantly patients suspected of leptospirosis proved to have suffered from hantavirus infections. This confirms that the primary differential diagnosis for

leptospirosis is NE and vice versa. In contrast to other countries [Glass et al., 1990, 1993; Tsianos et al., 1993] no serological evidence of hantavirus infection was found in haemodialysis patients. In some seronegative patients however, hantavirus antigen was demonstrated in the kidneys (chapter 2). The absence or barely detectable levels of hantavirus specific antibodies in hantavirus infected individuals has been reported before [Pether and Lloyd, 1993] and was also shown in one of the monkeys which we had infected experimentally with Puumala virus (chapter 4).

In The Netherlands like in other countries, a certain occupational risk of acquiring hantavirus infections was shown to exist in foresters, farmers and animal trappers (chapter 3). This is in agreement with the observation that most of the hantavirus infections occur in forested and rural areas of the country. Another interesting finding, which is also observed in other European countries, is the predominance of males between 30 and 40 years of age among the Puumala virus infected individuals which is reminiscent of the predominance of Hantaan virus infections in males between 20 and 50 years in Asia [Tkachenco and Lee, 1991]. It is highly unlikely that this is a reflection of the exposure rate of this category of individuals, since it may be speculated that individuals of younger age groups are at least exposed with the same frequency. Although on basis of our data, it may be speculated that the older age groups are more suspectible to developing disease symptoms upon hantavirus infection, this is not very likely, since Scandinavian studies have shown that the overall pattern of seropositivity also shows a peak in the same age category [Niklasson et al., 1987].

Virtually all the hospitalized NE cases in The Netherlands run a relatively mild course, with a clinical pattern similar to the cases of NE described in the neighbouring and Scandinavian countries [Settergren et al., 1989; Van Yepersele De Strihou and Mery, 1989]. It is remarkable, that the relatively minor histological changes observed in kidney biopsies (chapter 2), are associated with such a dramatic clinical course: The transient massive proteinuria and oliguria/anuria followed by polyuria would suggest more histological changes to be present.

In Western Europe hantavirus antibodies and antigen have also been detected in non-rodent species, like bats and cats [Bennet et al., 1990; Clement et al., 1994]. Screening of domestic and feral animals for the presence of hantavirus antibodies and/or antigens in The Netherlands only demonstrated the presence of hantavirus infections in red bank voles (*Clethrionomys glareolus*), the common shrew (*Crocidura russula*) and the common vole (*Microtus arvalis*), which all proved to be infected with Puumala-like virus. However, it should be stressed that the majority of the rodents had been trapped in areas where human cases of NE had been identified (chapter 3), and that the collection of sera from feral and domestic mammals screened, can not be considered a representative sample of the respective populations

in The Netherlands. Like in the neighbouring countries the red bank vole seems to be the main reservoir host of the Puumala-like virus in The Netherlands (chapter 3).

The fact that most of the clinical cases of NE in The Netherlands, which have been identified to date, have been diagnosed retrospectively by serology or immunohistochemistry, clearly indicates that the diagnosis NE is often overlooked by clinicians. This also indicates that it is difficult to estimate the true incidence of hantavirus infections in The Netherlands. Furthermore, it should be realized that subtle changes in the ecosystem of the rodents involved may lead to dramatic changes in prevalence of human hantavirus infections, as occurred during the recent epidemic of HPS amongst the Navajo indians in the USA [CDC, 1993].

Development of a non-human primate model

To date little is known about the pathogenesis of hantavirus infections in humans and it is particularly puzzling why the minor changes observed in the kidneys may lead to acute renal failure. This lack of understanding is largely due to the absence of an adequate animal model to study the early events of the human infection. This stage of the infection can hardly be studied, since patients are usually recognised and not admitted to the hospital before severe clinical signs have developed. Although rodent models have extensively been explored, these do not allow studies mimicking the pathogenesis of hantavirus infections in humans, since the pathogenesis in rodents is quite different. However, rodent models may provide insight in the mechanisms underlying persistent infection of reservoir host, which leads to direct or indirect exposure of humans. Laboratory mice have been used to study the pathogenicity of infections with different subtypes of hantavirus. Lethal infections were observed upon infection of newborn mice with Hantaan virus (strain 76-118) via different routes [McKee et al., 1985]. The red bank vole can be infected experimentally with Puumala virus, which leads to an inapparent chronic infection [Yanagihara et al., 1985], characterized by a transient viraemia and virus persistence particularly in the lungs, without causing any clinical or histological changes [McKee et al., 1985].

Possibilities for passive and active immunization against hantavirus infection have been studied in rodents and humans. Passive immunization of rats with polyclonal rat antibodies demonstrated complete protection against the homologous virus. Cross protection was found with viruses of the same subtype and only partly with viruses from different subtypes [Zhang et al., 1989]. In Korea an inactivated vaccine produced in the brains of suckling rodents proved to protect mice against challenge with the homologous Hantaan virus [Lee et al., 1990]. The same vaccine has also

been administrated to more than 500 human individuals, which resulted in 99% seroconversion. Although the investigators claimed that this vaccine is safe, side effects were noted [Lee et al., 1990]. Humoral and cellular immunity and no side effects were demonstrated in volunteers immunized with a Hantaan virus vaccine produced in cell culture [Song et al., 1991]. Although candidate vaccines against hantavirus infection have been tested for safety and efficacy in rodent models for use in humans, their evaluation in animal species which upon infection develop the same pathogenesis as humans, would be of greater value. Thus, not only for the study of the pathogenesis but also to study intervention strategies like vaccination and therapy, a relevant animal model would be of great value. Therefore we decided to explore the possibilities to develop a non-human primate model for hantavirus infection. We have chosen for the cynomolgus macaque, primarily since this species has been used in our laboratory extensively for the study of other human virus infections. These included infections with polio-, papova-, measles- , influenza- and lentiviruses [Osterhaus and Van Steenis, 1981; Van Steenis et al., 1983; Van Binnendijk et al., 1994; Hulskotte et al., 1995; Rimmelzwaan et al., 1995]. This lead to the establishment of assay systems which allow studies of the kinetics of the respective infections, but also of the virus specific antibody and T-cell responses. Puumala virus was chosen, since this virus is most prevalent in North Western Europe and was expected to cause relatively mild symptoms in this primate species. We decided to infect the macaques intratracheally since this probably most closely mimics the natural aerosol infection in humans. Only mild clinical symptoms were observed in the infected monkeys. It should be stressed however, that also in humans the common outcome of infection with Puumala virus is rather mild. This may also be concluded from the epidemiological studies presented in chapter 3. In fact the serological parameters measured upon infection, largely paralleled those found in humans, with the exception of the pattern of virus specific antibody subclasses, which are partly different in monkeys [Gaarder and Natvig, 1974; Van Loghem and De Lang, 1979]. Moderate histological changes could be demonstrated in the kidneys of the monkeys during the acute stage of the infection, which is also in agreement with histological findings in biopsies of human kidneys taken during the acute stage (chapter 2). The presence of viral antigen and RNA, as demonstrated by immunohistochemistry and nucleic acid hybridization, also followed the kinetics observed in humans (chapter 2) [Grankvist et al., 1992; Kim et al., 1993; Pojak and Avsic-Zupanc, 1994]. A lot has been speculated about persistence of hantavirus infections in humans. The monkey data presented do not support these speculations, since detectable levels of viral antigen and RNA disappeared from the sofar limited number of monkey kidney studied. The extreme difficulty to isolate Puumala virus from the kidneys of experimentally infected monkeys is reminiscent of the situation

in infected humans. Although three subsequent passages were carried out in different cell substrates, all of which had been shown to be permissive for Puumala virus replication, the virus was not isolated from monkey kidneys. It should however be realized that more extensive protocols have eventually yielded success with human specimens [Grankvist et al., 1992].

Concluding remarks

The results of the studies presented in this thesis show that Puumala virus infections are an important cause of acute renal disease in certain areas in The Netherlands and that infection with this virus is much more prevalent than may be estimated from the limited number of diagnosed clinical cases. Although the areas involved seem to be rather constant over time, it should be born in mind that changes in the population dynamics of the rodent host species may have a major impact on the geograpical distribution and incidence of the disaese. This was recently demonstrated during outbreaks of hantavirus infections in Europe and the USA [CDC, 1993; Clement et al., 1994; Le Guenno 1994] (chapter 3). The most likely sources of human hantavirus infections in The Netherlands are direct or indirect contacts with the red bank vole (chapter 3). The establishment of rapid sensitive and specific diagnostic methods for Puumala virus infection based on serological, immunohistochemical and molecular biological techniques as presented, created the tools for the diagnostic and epidemiological studies carried out in The Netherlands in the context of this thesis. Finally, it may be concluded that the first description of a primate model for a hantavirus infection, may not only lead to the in depth study of the pathogenesis, but also to new possibilities for vaccination and therapeutic approaches, for Puumala virus and other hantavirus infections.

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Nederlandse samenvatting

Haemorrhagische koorts met renaal syndroom veroorzaakt door infecties met hantavirussen kwam voor het eerst onder de aandacht van de westerse medici tijdens de Koreaanse oorlog van 1951 - 1953. Tijdens deze oorlog deed zich een epidemie voor onder circa 3000 VN militairen. De infectie werd gekenmerkt door koorts, bloedingen, shock en nierfunctiestoornis met een mortaliteit oplopend tot ca. 15%. Het virus dat dit ziektebeeld veroorzaakte werd pas 25 jaar later door dr. H.W. Lee en zijn medewerkers geïsoleerd uit de Koreaanse veldmuis (*Apodemus agrarius*) en werd genoemd naar de Hantaan rivier. Dit virus was voor die tijd nog nooit eerder uit mensen of dieren geïsoleerd en werd gekarakteriseerd als een nieuw virus, dat werd ingedeeld in een nieuw genus van de *Bunyaviridae* familie. Het Hantaan virus werd het prototype van dit genus. Het genus *Hantavirus* is het enige genus van deze familie dat niet door stekende of bijtende insekten op de mens wordt overgebracht, maar door direct of indirect contact met besmette knaagdieren. Al gauw werd duidelijk dat het voorkomen van dit virus niet beperkt was tot Korea.

In dit proefschrift worden nieuwe diagnostische systemen beschreven, voor de detectie van hantavirusinfecties bij mens en dier. Deze testsystemen worden gebruikt om het klinisch beloop en de epidemiologie van hantavirusinfecties in Nederland te bestuderen.

De algemene inleiding - hoofdstuk 1 - geeft een overzicht van de karakteristieke eigenschappen van hantavirussen en de ziektebeelden die door de verschillende subtypen van dit virus worden veroorzaakt. In het tweede gedeelte van dit hoofdstuk wordt aandacht besteed aan de laboratoriumdiagnostiek en de verspreiding van hantavirusinfecties bij mens en dier.

Het acute ziektebeeld dat door een hantavirusinfectie wordt veroorzaakt, wordt in Nederland vaak niet als zodanig herkend. Voor de diagnose van deze infectie is men vooral aangewezen op serologische testsystemen. In hoofdstuk 2 worden allereerst verschillende enzyme-linked immunosorbent assays (ELISA) beschreven, gebaseerd op verschillende principes, waarmee snel en relatief eenvoudig hantavirus-specifieke antistoffen in serum of plasma en hantavirus antigeen in organen kunnen worden aangetoond. Deze ELISA worden vergeleken met de veel gebruikte immunofluorescence assay (IFA). In die gebieden waar het voorkomen van hantavirus-specifieke antistoffen onder de bevolking relatief hoog is - variërend van 0.5 tot 2% - kan men voor het stellen van de diagnose geen gebruik maken van standaard testsystemen gebaseerd op het aantonen van IgG specifieke antistoffen in het serum. Daarom werden verschillende andere testsystemen ontwikkeld (hoofdstuk 5, tabel 1, pagina 147) om de tijd na de aanvang van symptomen vast te stellen. In het acute stadium bleken hantavirus-specifieke IgA, IgM en IgG3 antistoffen aantoonbaar te zijn.

Enkele weken tot maanden na infectie verdwijnen deze antistoffen veelal en kunnen alleen nog maar specifieke IgG1 antistoffen aangetoond worden. Gebruikmakend van monoclonale antistoffen gericht tegen de groep specifieke antistofbindingsplaatsen (epitopen) van het virus, op glycoproteïne G1, G2 en het nucleoproteïne, werden met behulp van een competitie ELISA de antistofpatronen in het serum van de patiënten bepaald op verschillende tijdstippen na infectie. Dit testsysteem toonde aan dat in het acute stadium van de infectie hoge antistofwaarden tegen het virale glycoproteïne G1 en het nucleoproteïne in het serum van de patiënt aanwezig waren. Enkele weken na de infectie werden hoge antistofwaarden in het serum gemeten tegen het virale glycoproteïne G2. Dit patroon vertoonde gelijkenis met het patroon van de specifieke IgG1 antistoffen. Bij enkele van deze patiënten werd tijdens het acute stadium van de infectie een nierbiopt genomen. Met behulp van de monoclonale antistoffen kon hantavirus-specifiek eiwit in het nierweefsel van deze patiënten worden aangetoond.

In hoofdstuk 3 wordt een aantal van de in hoofdstuk 2 beschreven ELISA systemen gebruikt om het voorkomen van hantavirusinfecties bij mensen en dieren in verschillende gebieden in Nederland te onderzoeken. Het eerste onderzoeksprogramma naar het voorkomen van deze infectie werd uitgevoerd met het in 1976 ontdekte Hantaanvirus 76-118. Vanaf 1984 werden er wereldwijd verschillende hantavirus subtypen geïsoleerd, waaronder het Seoulvirus, het Puumalavirus en het Prospect-Hill virus. De in hoofdstuk 2 beschreven IFA en ELISA systemen voor de diagnose van hantavirusinfecties werden aangepast om tegen deze subtypen antistoffen te kunnen aantonen. Deze subtype specifieke ELISA systemen werden gebruikt om het voorkomen van verschillende hantavirus subtypen bij mensen in Nederland en België te onderzoeken. Retrospectief onderzoek van serummonsters van individuen met een klinisch bewezen hantavirusinfectie toonde bij een aantal laboratoriummedewerkers van het Rijksinstituut voor Volksgezondheid en Milieuhygiëne te Bilthoven, alsmede van een instituut in Brussel, hoge antistofwaarden aan tegen Hantaan- en Seoul-achtige virussen. Dit in tegenstelling tot het merendeel van de individuen die niet werkzaam waren op een laboratorium en waarbij in het serum antistoffen werden aangetoond tegen Puumala-achtige virussen die in Nederlandse knaagdieren voorkomen. Bij slechts twee individuen - één uit Nederland en één uit België - werden hogere antistofwaarden aangetoond tegen Hantaan- en Seoul-achtige virussen. Dit zou erop kunnen wijzen dat er in Nederland en België meerdere hantavirus subtypen voorkomen. Om de geografische verspreiding van hantavirusinfecties in Nederland en het reservoir van dit virus te identificeren, werden in de periode van 1984 tot 1994, serummonsters van individuen met en zonder een nierlijden, individuen met een mogelijk beroepsrisico, gezelschapsdieren en in het wild levende dieren, afkomstig van verschillende locaties in ons land, onderzocht. Uit dit onderzoek bleek dat het merendeel van de hantavirus-

seropositieve individuen met of zonder klinische symptomen, woonachtig of werkzaam waren in landelijke of bosrijke omgevingen in het oosten en zuiden van ons land. Tevens bleek uit dit epidemiologisch onderzoek dat er een beroepsrisico bestaat voor onder meer bosarbeiders, boeren en medewerkers van plaagdierenbestrijdingsdiensten. Van de 34 onderzochte diersoorten werden hantavirus-specifieke antistoffen of -antigeen aangetoond bij de rosse woelmuis *(Clethrionomys glareolus)*, de gewone spitsmuis (*Crocidura russula*) en de veldmuis (*Microtus arvalis*). De rosse woelmuis bleek de belangrijkste reservoir gastheer in Nederland te zijn.

Hoofdstuk 4 beschrijft de ontwikkeling van een apenmodel voor hantavirusinfecties. Dit diermodel is vooral van belang om inzicht te krijgen in de pathogenese van hantavirusinfecties en voor de evaluatie van diagnostische testsystemen, vaccin ontwikkeling en antivirale therapie. Voor dit onderzoek zijn Java apen via de luchtwegen besmet met het in Nederland voorkomende hantavirus (Puumala-achtig). Evenals bij de mens werden in deze apen slechts milde klinische symptomen waargenomen. Hantavirus specifieke antistofpatronen aangetoond in deze dieren toonden grote overeenkomst met patronen die bij mensen met een Puumalavirusinfectie gezien werden. Tevens werden geringe histologische afwijkingen en hantaviruseiwit in de nieren van deze dieren aangetoond. Dit komt eveneens overeen met de waarnemingen in nierbiopten van hantavirus patiënten in ons land.

In hoofdstuk 5 worden de resultaten van dit proefschrift samengevat en verder bediscussieërd. De ontwikkelde snelle en gevoelige testsytemen voor het aantonen van hantavirus gebaseerd op serologische, immunohistochemische en moleculair biologische technieken vormden de basis voor de diagnostische en epidemiologisch studie uitgevoerd in Nederland. Deze studie toonde aan dat Puumalavirusinfecties in bepaalde gebieden in Nederland een belangrijke oorzaak zijn van acute nierfunctiestoornis. Tevens bleek dat dergelijke infecties frequenter voorkomen dan kan worden opgemaakt uit het relatief geringe aantal klinisch gediagnostiseerde gevallen bij de mens. Tenslotte wordt geconcludeerd dat deze eerste beschrijving van een apenmodel voor Puumalavirusinfecties wellicht zal leiden tot nader onderzoek naar de pathogenese van andere hantavirus subtypen, terwijl zij tevens de mogelijkheid zal bieden om vaccinaties en therapie-experimenten uit te voeren voor infecties met Puumalavirus en andere hantavirus subtypen.

Dankwoord

Dit is naar alle waarschijnlijkheid het meest gelezen hoofdstuk van het hele proefschrift (voor de een omdat hij/zij in dit hoofdstuk niets aantreft over de inhoud van het proefschrift en voor de ander om te kijken of hij/zij al dan niet genoemd wordt). Promovendi pretenderen vaak alles zelf te hebben bedacht, onderzocht en geschreven. Ik ook! Het dankwoord schetst veelal een ander beeld. Zie het mijne.

Ik hoop met mijn dankwoord een beeld te kunnen geven van de sfeer, de vele collega's, vrienden en familie die het raamwerk hebben gevormd bij het tot stand komen van dit proefschrift. Ik wil dan ook iedereen bedanken die de afgelopen jaren deel hebben genomen aan dit proces.

Ab, op mijn eerste werkdag in augustus 1984 kreeg ik mijn eerst dosis Albert Stimulerend Complex (ASCOM) reeds toegediend. De tweede volgde na mijn proeftijd van twee maanden, gevolgd door een derde bij mijn vaste aanstelling weer twee jaren later. Mijn enthousiasme werd in ieder geval door jou versterkt. Ik vraag me af hoe hoog de dosis was die jij gebruikte en nog steeds gebruikt. Waarschijnlijk is dat een dagelijkse shot. Jouw enthousiasme en de inzet en energie, waarmee jij je leven inhoud geeft en jij je hebt ingezet bij het tot stand komen van dit proefschrift zullen mij altijd bijblijven. Ook de stimulerende complexen (S & W) van Roos, veelal oraal toegediend, hadden op mij de afgelopen jaren een goede uitwerking en ik hoop in de toekomst hiervan nog veel gebruik te maken.

Prof. dr. M. Horzinek, Prof. dr. G. van der Groen en Prof. dr. J. van der Noorda wil ik bedanken voor het vertrouwen dat zij mij gaven om tot promotie te worden toegelaten. Guido van der Groen, jou wil ik in het bijzonder bedanken. Niet zozeer uit affectie, omdat je een "aangeklede" naamgenoot bent, maar voor je commentaar op dit proefschrift. Ik zal overigens nooit vergeten dat je mij, tijdens mijn eerste Hantavirus-congres in 1987 te Antwerpen, erop wees dat het genus *Hantavirus* ook nog tot de sub-familie behoort van de *Hantaviridae*-freaks met species als Joel Dalrymple, Jim Le Duc, Ho-Wang Lee, Tantjana Avsic Zupanc, Conny Schmaljohn, Bernard Le Guenno, Jan Clement en vele andere.

Mijn dank gaat ook uit naar de medewerkers van het Medisch Spectrum Twente in Enschede. In het bijzonder gaat mijn dank uit naar Hein Jordans en Martin Gerding. Jullie patiëntenbestand vormde voor een groot deel de basis van dit proefschrift.

Dr. G. van Steenis (RIVM) wil ik bedanken voor de prettige en inspirerende sfeer,

waarin ik heb kunnen werken. Ditzelfde geldt ook voor dr. Ph.H. Rothbarth (AZR, virologie) en zijn medewerkers, waarmee ik nu samenwerk.

De lange militaire geschiedenis van Hantavirusinfecties heeft op dit proefschrift een stempel gedrukt. Hierbij wil ik dan ook de volgende officieren "onderscheiden" met mijn hartelijke dank voor hun medewerking: Kolonel Hans Bruins (DMGZ), Kolonel Jan Clement (Militair Hospitaal Brussel), Majoor Wim Wertheim (DMGZ), Luitenant Eric Rooijakkers (ROAG) en tot slot Vaandrig Frank Pistoor. Soldaat 1e klas buitendienst Wim Murk hoort eigenlijk ook tot de officieren. Wat ik van jou heb gehoord, Wim, is met geen pen te beschrijven. Ik waag me daar dan ook niet aan. Tevens wil ik alle eigenaren van de biologische slagvelden, met name in de regio Twente, bedanken voor het beschikbaar stellen van de grond voor de veldtroepen. Deze veldtroepen wil ik een speciale onderscheiding geven voor het plaatsen en onschadelijk maken van de "mouse traps". De veldtroepen werden geformeerd door Heinz Broeders, Bart van Ordell, Alex Lefevre, Martin Gerding, Ton Brink en Ab Osterhaus. **Mannen bedankt!** Dit geldt ook voor de medewerkers van de sociale en experimentele divisies van de thuisbasissen (RIVM en AZR): Gerben Drost, Paul Roholl, Joke Robinson, Byron Martina, Adri Vermeulen, Ruud van Kinderen, Kees Siebelink, Guus Rimmelzwaan, Jos Karlas, Robin Huisman, Rik de Swart, Carel van Baalen, Eric Claas, Heinz Broeders, Rob van Binnendijk, Marco van de Bildt, Helma Vos, Roger van der Heijden, Fons UytdeHaag, Bert Niesters, Edwin Vries en Jans Velzing. **Vrouwen en mannen bedankt!** Een speciale eenheid binnen de divisies werd gevormd door soldaat eerste klas Geert van Amerongen en soldaat tweede klas Nico Schmidt. Hun vindingrijkheid, bereidwilligheid en verrassingstactiek gaven een speciaal effect aan mijn onderzoek. **Mannen bedankt!** Ook ons "enige" pantservoertuig met registratie code RE-GA-TA en de transportmanschappen waren onmisbaar. Heinz Broeders, Peter Wijntjes en Kees Siebelink. **Mannen bedankt!**

Cc. staat niet voor correspondance copy, maar binnen onze afdeling voor controlled by Conny. Conny bedankt voor de correcties van mijn papers. Jeanet, jou wil ik bedanken voor het overtypen voor een deel van mijn papers, die door cc. en mijzelf niet meer op flop waren bewaard. Guus onze samenwerking is meestal één groot hoogtepunt. Gelukkig, meestal niet in het lab.

Al het epidemiologisch onderzoek was onmogelijk geweest zonder de vrijwillige bijdrage van vele patiënten en de steun van de medewerkers van: de Bloedbank Twente, het Academisch Ziekenhuis Utrecht (afdeling Nierdialyse), het Academisch Ziekenhuis Maastricht (afdeling Immunologie), het Academisch Ziekenhuis Leiden (laboratorium voor Pathologie), de stichting Thuisdialyse Utrecht, de Plaagdier Bestrijdingsdienst, Staatsbosbeheer, de Bedrijfsgezondheidsdienst RBB, het Koninklijk

Instituut voor de Tropen, het Centraal Diergeneeskundig Instituut, de Veterinaire Hoofdinspectie (in het bijzonder dr. J. Nieuwenhuijs) en vele andere instellingen.

Mede door de basis die ik van pa en ma heb meegekregen heb ik dit kunnen bereiken. Gerda, ik ben blij dat je er bij bent. Nol en Jo, jullie feestkleding kan eindelijk uit de mottenballen.

Lieve Charlotte, mijn eerste artikel voor dit proefschrift schreef ik in onze "suite" in de Jan Tooropstraat te Amsterdam. Nu, enkele jaren verder en in het gelukkige bezit van onze twee kinderen Levi en Roos, wil jou bedanken voor het geluk, de liefde en de gezelligheid, waarmee je mij omringt.

Collega's, vrienden en familie bedankt.

Jan

Curriculum vitae

De auteur van dit proefschrift is geboren op 1 december 1959 te Amersfoort. Na de middelbare school begon hij in 1975 met de middelbare beroepsopleiding tot chemisch analist te Amersfoort. Hierna volgde hij de hogere beroepsopleiding tot botanisch analist aan de stichting tot opleiding van analisten (STOVA) te Wageningen. De eerste drie jaren, nadat zijn opleidingen in 1981 waren afgerond, werkte hij als analist bij Gist-Brocades N.V. in De Bilt. In de periode 1984 tot 1993 was hij in dienst van het Rijks Instituut voor Volksgezondheid en Milieuhygiëne te Bilthoven. De eerste drie jaren als researchmedewerker van de afdeling animale virologie en later als wetenschappelijk onderzoeksmedewerker van de afdeling virale zoönosen van het Laboratorium voor Immunobiologie. Gedurende deze periode werden verschillende stages en opleidingen gevolgd in binnen- en buitenland ter vergroting van zijn ervaring met virologisch en immunologisch onderzoek aan vooral exotische virusinfecties. Sinds 1 januari 1993 is de auteur werkzaam als wetenschappelijk medewerker bij het Instituut voor de Virologie van het Academisch Ziekenhuis Rotterdam.

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