SATU MÄKELÄ

Determinants of Clinical Course and Outcome of Puumala Hantavirus-induced Nephropathia Epidemica

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ACADEMIC DISSERTATION

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To my family

ABSTRACT

Nephropathia epidemica (NE) is a mild type of hemorrhagic fever with renal syndrome caused by Puumala hantavirus. NE presents with fever, headache, gastrointestinal symptoms, and impaired renal function. The course of NE varies from asymptomatic to fatal.

The tumor necrosis factor(TNF) α(-308) allele 2 (TNF2) is known to be in linkage disequilibrium with the HLA-B8-DRB1*0301(DR3) haplotype. Both factors have previously been associated with severe NE. To examine which part of this extended haplotype might show the strongest association with the outcome of NE, the HLA-B, -DRB1 and TNFα(-308) alleles were analyzed in 116 hospital-treated patients. The findings pointing to clinically severe disease were strongly associated with HLA-B8-DR3 haplotype. There was also a trend towards a severe disease in TNF2-carriers. This was probably due to linkage disequilibrium with B8-DR3, since there were no differences in the clinical severity when TNF2-positive/B8-DR3-negatives were compared to TNF2-negative/B8-DR3-negatives.

The genotypes of the genes of TNFα, interleukin-1α (IL-1α), IL-1β and IL-1 receptor antagonist (IL-1Ra) were analyzed by PCR in 87 hospital-treated patients with NE to establish whether gene polymorphisms are associated with susceptibility to NE. The frequency of non-carriers of IL-1Ra allele 2/IL-1β(-511) allele 2 was shown to be increased in patients compared to seronegative controls. Further, the plasma levels and overnight urinary excretions of IL-1β, IL-1Ra, IL-6 and TNFα were observed to be markedly increased in 70 hospitalized NE patients. Urinary IL-6 showed a positive correlation with urinary albumin, but none with plasma IL-6.

46 previously healthy subjects who had suffered NE 3-7 years previously were studied to establish the long-term prognosis of NE. The patients evinced higher mean ambulatory systolic blood pressure, higher GFR (determined by ^{51}CrEDTA clearance), and more proteinuria than the seronegative controls. Five patients who developed mesangiocapillary glomerulonephritis during the convalescent phase of NE were also described.

In conclusion, the HLA-B8-DR3 haplotype is associated with a severe clinical course of NE. The urinary cytokines, notably IL-6, might be locally produced in the kidneys during acute infection. The prognosis of NE is favorable. Nevertheless, the infection may cause mild tubular lesions and hypertension in some patients.

CONTENTS

ABBREVIATIONS

ABP	ambulatory blood pressure
BMI	body mass index
C	complement factor (e.g., C3)
CRP	C-reactive protein
CTL	cytotoxic T-lymphocyte
DBP	diastolic blood pressure
DIC	disseminated intravascular coagulation
DNA	deoxyribonucleic acid
DOBV	Dobrava virus
ELISA	enzyme-linked immunosorbent assay
ERPF	effective renal plasma flow
FF	filtration fraction
G1, G2	glycoproteins (of hantaviruses)
GFR	glomerular filtration rate
HFRS	hemorrhagic fever with renal syndrome
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPS	hantavirus pulmonary syndrome
HTNV	Hantaan virus
IFA	immunofluorescence assay
IFN	interferon
Ig	immunoglobulin
IL	interleukin
MBP	mean blood pressure
MCGN	mesangiocapillary glomerulonephritis
N	nucleocapsid protein (of hantaviruses)
NE	nephropathia epidemica
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PUUV	Puumala virus
Ra	receptor antagonist (e.g., IL-1Ra)
RNA	ribonucleic acid
SAAV	Saaremaa virus
SBP	systolic blood pressure
SEOV	Seoul virus
SLE	systemic lupus erythematosus
SNV	Sin Nombre virus
TGF	transforming growth factor
TNF	tumor necrosis factor

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following five original studies, which are referred to in the text by their Roman numerals I-V.

I Mäkelä S, Hurme M, Ala-Houhala I, Mustonen J, Koivisto A-M, Partanen J, Vapalahti O, Vaheri A, Pasternack A (2001): Polymorphism of the cytokine genes in hospitalized patients with Puumala hantavirus infection. Nephrol Dial Transplant 16: 1368-73.

II Mäkelä S, Mustonen J, Ala-Houhala I, Hurme M, Partanen J, Vapalahti O, Vaheri A, Pasternack A (2002): Human leukocyte antigen-B8-DR3 is a more important risk factor for severe Puumala hantavirus infection than the tumor necrosis factor- alpha(-308) G/A polymorphism. J Infect Dis 186: 843-6.

III Mäkelä S, Mustonen J, Ala-Houhala I, Hurme M, Koivisto A-M, Vaheri A, Pasternack A: Urinary excretion of interleukin-6 correlates with proteinuria in acute Puumala hantavirus infection (submitted).

IV Mäkelä S, Ala-Houhala I, Mustonen J, Koivisto A-M, Kouri T, Turjanmaa V, Vapalahti O, Vaheri A, Pasternack A (2000): Renal function and blood pressure five years after Puumala virus-induced nephropathy. Kidney Int 58: 1711-8.

V Mustonen J, Mäkelä S, Helin H, Helanterä A, Miettinen M, Partanen J, Pasternack A (2001): Mesangiocapillary glomerulonephritis caused by Puumala hantavirus infection. Nephron 89: 402-7.

In addition, this thesis contains unpublished data.

INTRODUCTION

Hantaviruses are found worldwide and in many areas are associated with significant mortality and morbidity among humans. They cause two human zoonoses. Hemorrhagic fever with renal syndrome (HFRS) is caused by Hantaan, Dobrava, Puumala, Saaremaa and Seoul viruses, while Sin Nombre and several other hantaviruses found in North and South America cause hantavirus pulmonary syndrome (HPS). Some 150,000 HFRS cases are estimated to occur per year worldwide (Vapalahti et al. 2003). HPS occurs in the Americas with over 1,000 cases reported since 1993, when the disease was first described.

A mild form of HFRS, named nephropathia epidemica (NE) occurs widely in Finland, Scandinavia, western Russia and several other areas in Europe. The causative agent, Puumala virus (PUUV), is a member of the genus *Hantavirus* in the *Bunyaviridae* family. Its natural host is the bank vole *(Clethrionomys glareolus)*. In Finland, approximately 1,000 serological diagnoses are made annually and the seroprevalence of NE in the population is 5% (Brummer-Korvenkontio et al. 1999). NE is clinically characterized by high fever, headache, nausea, vomiting, abdominal and back pain and blurred vision. Renal involvement results in transient proteinuria, hematuria and impairment of renal function followed by polyuria and spontaneous recovery (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a). The typical histopathological renal finding is acute tubulointerstitial nephritis (Mustonen et al. 1994b). Common laboratory findings are leukocytosis, thrombocytopenia and elevation of serum C-reactive protein (CRP) and creatinine levels (Settergren et al. 1989, Mustonen et al. 1994a). There is considerable variability in the clinical severity of NE, ranging from asymptomatic to occasionally fatal disease. There is evidence that host genetic factors are involved in the clinical picture (Mustonen et al. 1996). The prognosis of NE is considered favorable, but occasional reports

of mild tubular dysfunction and hypertension after HFRS have been described (Lähdevirta et al. 1978, Glass et al. 1993).

HFRS is formally recognized by the World Health Organization (WHO 1983) as a public health problem and hantaviral diseases are also included among the so-called emerging infections (Schmaljohn and Hjelle 1997). Consequently much effort is invested internationally in research on their virology, epidemiology and pathogenesis, as well as on vaccine development.

In this series, the influence of host genetic factors on the highly variable clinical course of acute Puumala hantavirus infection was studied. The outcome of NE three to seven years after acute disease was also evaluated. Such clinical data might be of value in assessing the need for follow-up visits after NE or in devising vaccination programs.

REVIEW OF THE LITERATURE

Puumala virus and other hantaviruses

History

As early as 1913, clinical records from Far East Russia described an HFRS-like disease (Lee 1982). However, a similar disease is already to be found in a Chinese medical account as early as 960 AD. Japanese military physicians encountered the diseased after the invasion of Manchuria in 1934 and described the clinical symptoms and pathology in detail. Already then, rodents were suspected as possible reservoirs of the etiological agent. In Europe, an HFRS-like disease was described by two Swedish physicians, independently of each other (Myhrman 1934, Zetterholm 1934). Myhrman named the disease nephropathia epidemica (Myhrman 1951). During World War II, Finnish and German troops encountered a similar disease in Finnish Lapland, the outbreak coinciding with a high population density of rodents. The most famous outbreak of an HFRS-like disease occurred during the Korean conflict in 1951-1953, when about 3,000 American and Korean soldiers were severely stricken with an illness characterized by renal failure, hemorrhages and shock (Lee 1982). The disease was named Korean hemorrhagic fever in Korea and epidemic hemorrhagic fever in China and Japan (Lee and van der Groen 1989). By the early 1960s, the similarity between the HFRS-like diseases seen in East Russia and Asia to those occurring in Western Russia and Europe was noted, and it was suggested that they were caused by related agents (Gajdusek 1962).

The infecting agent remained however unknown until 1976, when Dr. Ho Wang Lee employed an immunofluorescence assay (IFA) to detect viral antigens in infected rodent tissue by the use of patient sera (Lee and Lee 1976). Hantaan virus (HTNV) was subsequently isolated from a striped field mouse (*Apodemus agrarius*) captured near the Hantaan river in South Korea (Lee et al. 1978), and by a similar approach another agent from urban rats (*Rattus norvegicus and R. rattus*) in Seoul, Korea, was also found to cause disease (Lee et al. 1982). IFA was also used by Brummer-Korvenkontio and colleagues (1980), who reported that tissue samples from bank voles (*Clethrionomys glareolus*) reacted with sera from NE patients. The virus, first detected in samples collected near the village of Puumala in Finland in 1977 (Brummer-Korvenkontio et al. 1980), was subsequently isolated in colonized bank voles and named Puumala virus (Schmaljohn et al. 1985). The term "HFRS", as suggested by Gajdusek (Gajdusek 1962), was adopted by the WHO in 1983 (WHO 1983). In 1983, analysis of HTNV ribonucleic acid (RNA) revealed that these viruses belonged to the *Bunyaviridae* family and formed a new *Hantavirus* genus (Schmaljohn and Dalrymple 1983). The first and prototypic American hantavirus, Prospect Hill virus, was isolated from meadow voles in the USA in 1982 (Lee et al. 1985).

HPS was first described in 1993 when a cluster of fatal cases of an acute respiratory distress of unknown origin occurred in the Four Corners area of the United States (New Mexico, Arizona, Colorado, and Utah). The finding that sera from patients reacted with hantaviral antigens was quickly followed by the genetic identification of a novel hantavirus in patients' tissues and in rodents trapped near patients' homes (Nichol et al. 1993, Ksiazek et al. 1995, Schmaljohn et al. 1995). The causative agent was first named Four Corner virus, but this was later changed to Sin Nombre virus (virus with no name), which did not refer to any particular geographic region (Schmaljohn et al. 1995).

Virology

Hantaviruses are enveloped RNA viruses belonging to the family *Bunyaviridae* (Schmaljohn et al. 1985). They have a negative-stranded RNA genome with

three segments. The large RNA segment encodes the viral RNA-polymerase, which is believed to be responsible for all steps of transcription and replication of the viral genome (Plyusnin 2002). The medium segment encodes two envelope glycoproteins, G1 and G2, which are thought to recognize hantavirus receptor(s) on target cells. The small segment of hantaviruses encodes a nucleocapsid protein (N) (Schmaljohn et al. 1985). The N and glycoproteins both evoke antibody responses and induce protective immunity (Schmaljohn et al. 1990, Lundkvist et al. 1993b).

Each of the currently recognized hantavirus species is predominantly associated with one (or a few closely related) specific rodent species (Plyusnin et al. 1996b, Plyusnin and Morzunov 2001, Plyusnin 2002). It would appear that hantaviruses have co-evolved with their rodent hosts for millions of years, resulting in the circulation of distinct hantaviruses on different continents (Plyusnin et al. 1996b). Rodents harboring hantaviruses can be classified into three subfamilies of the family *Muridae,* namely the *Arvicolinae*, *Murinae* and *Sigmodontinae* (Elliott et al. 2000). Human hantavirus diseases manifest themselves in two clinical disease syndromes, HFRS and HPS. Hantaviruses from Eurasia, carried by *Arvicolinae* or *Murinae* rodents, can cause HFRS, while most of their relatives from the Americas, maintained by *Sigmodontinae* rodents, can cause HPS.

The persistently infected and probably asymptomatic carrier rodents may excrete the virus in their urine, saliva and feces for months (Lee et al. 1981, Bernshtein et al. 1999). The dried and aerosolized virus from these excreta probably infects humans via the respiratory tract (Lee et al. 1981, Tsai 1987). Transmission to human is apparently a dead-end for the virus. Virtually no evidence of person-to-person transmission of hantavirus has been noted (Vitek et al. 1996). There is, however, one report from Argentina of 16 Andes virus (ADNV)-induced HPS cases with no other suspected contacts beyond infected persons (Padula et al. 1998). In Finland, NE has been described in one 60-day-old breastfed baby whose mother had had the disease 25 days before the baby fell ill, and here person-to-person transmission is a possible explanation (Brummer-Korvenkontio et al. 1999).

To date, over 30 hantaviruses have been described, of which 22 have been accepted as species by the International Committee of Taxonomy of Viruses (Elliott et al. 2000), and the number is increasing due to intensified research in the field. In Table 1, hantaviruses, their rodent hosts and hantaviral clinical syndromes are listed. Several hantaviruses not associated with any human disease are also included.

Table 1*. Hantaviruses and their rodent hosts.

Virus (Abbreviation)	Principal rodent host	Known distribution	Disease	Ref.
Andes (ANDV)	Long-tailed pygmy rice rat (*Oligoryzomys longicaudatus*)	Argentina and Chile	HPS	(Lopez et al. 1996)
Bermejo	Chacoan pygmy rice rat (*Oligoryzomys chacoensis)*	Northwest Argentina	HPS	(Padula et al. 2002)
Lechiguanas	Rice rat *(Oligoryzomys flavescens)*	Central Argentina	HPS	(Levis et al. 1997)
Oran	Long-tailed pygmy rice rat *(Oligoryzomys longicaudatus)*	Northwest Argentina	HPS	(Levis et al. 1997)
Araraquara	Bolomys lasiurus?	Brazil	HPS	(Johnson et al. 1999)
Bayou (BAYV)	Rice rat *(Oryzomys palustris)*	South-eastern United States	HPS	(Morzunov et al. 1995)
Black Creek Canal (BCCV)	Cotton rat *(Sigmodon hispidus)*	United States, especially Florida	HPS	(Rollin et al. 1995)
Calapazo	Sugar-cane mouse *(Zygodontomys brevicauda)*	Panama	nd	(Vincent et al. 2000)
Caño Delgadito	Sigmodon alstoni	Venezuela	nd	(Fulhorst et al. 1997)
Castelo dos Sonhos	Unknown	Brazil	HPS	(Johnson et al. 1999)
Choclo	Pygmy rice rat *(Oligoryzomys fulvescens)*	Panama	HPS	(Vincent et al. 2000)
Dobrava (DOBV)	Yellow necked field mouse *(Apodemus flavicollis)*	Europe	HFRS	(Avsic-Zupanc et al. 1992)
Saaremaa (SAAV)	Striped field mouse *(Apodemus agrarius)*	Europe	HFRS	(Nemirov et al. 1999)
El Moro Canyon (ELMCV)	Western harvest mouse *(Reithrodontomys megalotis)*	United States, Mexico	nd	(Hjelle et al. 1994)
Hantaan (HTNV)	Striped field mouse *(Apodemus agrarius)*	China, Korea, Russia	HFRS	(Lee et al. 1978)
Isla Vista (ISLAV)	California meadow vole *(Microtus californicus)*	United States (California, Oregon), Baja (Mexico)	nd	(Song et al. 1995)

Juquitiba	Unknown	Brazil	HPS	(Johnson et al. 1999)
Khabarovsk (KHAV)	Reed vole *(Microtus fortis)*	East Russia	nd	(Hörling et al. 1996)
Laguna Negra (LANV)	Vesper mouse *(Calomys laucha)*	Bolivia and Paraguay	HPS	(Johnson et al. 1997)
Limestone Canyon	Brush mouse *(Peromyscus boylii)*	Western United States	nd	(Sanchez et al. 2001)
Maciel	Necromys benefactus	Central Argentina	nd	(Levis et al. 1997)
Muleshoe (MULV)	Cotton rat *(Sigmodon hispidus)*	Southern United States	nd	(Rawlings et al. 1996)
New York (NYV)	White-footed mouse (*Peromyscus leucopus*)	Eastern United States	HPS	(Song et al. 1994)
Pergamino	Grass field mouse *(Akodon azarae)*	Central Argentina	nd	(Levis et al. 1997)
Prospect Hill (PHV)	Meadow vole (*Microtus pennsylvanicus)*	North America	nd	(Rowe et al. 1995)
Bloodland lake (BLLV)	Prairie vole *(Microtus ochrogaster)*	North America	nd	Unpublished
Puumala (PUUV)	Bank vole *(Clethrionomys glareolus)*	Europe	HFRS	(Brummer-Korvenkontio et al. 1980, Schmaljohn et al. 1985)
Rio Mamore (RIOMV)	Small-eared pygmy rice rat *(Oligoryzomys microtis)*	Bolivia	nd	(Bharadwaj et al. 1997)
Rio Segundo (RIOSV)	Harvest mouse *(Reithrodontomys mexicanus)*	Costa Rica	nd	(Hjelle et al. 1995)
Seoul (SEOV)	Rat *(Rattus rattus, rattus norvegicus)*	worldwide	HFRS	(Lee et al. 1978)
Sin Nombre (SNV)	Deer mouse *(Peromyscus maniculatus)*	Western and Central United States, Mexico	HPS	(Nichol et al. 1993, Elliott et al. 1994)
Blue River	White-footed mouse *(Peromyscus leucopus)*	Central United States	nd	(Morzunov et al. 1998)
Monongahela	Cloudland deer mouse *(Peromyscus maniculatus nubiterrae*)	Eastern United States and Canada	HPS	(Song et al. 1996)
Thailand (THAIV)	Bandicoot rat *(Bandicota indica)*	Thailand	nd	(Xiao et al. 1994)
Thottapalayam (TPMV)	Musk shrew *(Suncus murinus)*	India	nd	(Carey et al. 1971)
Tobetsu	Bank vole *(Clethrionomys glareolus)*	Japan	nd	(Kariwa et al. 1995)
Topografov (TOPV)	Siberian lemming *(Lemmus sibericus)*	Asia (Siberia), Northern Europe	nd	(Plyusnin et al. 1996a)
Tula (TULV)	European common vole *(Microtus arvalis)*	Europe	nd	(Plyusnin et al. 1994)

*This table is adapted from Lednicky (Lednicky 2003) and the International Committee of Taxonomy of Viruses (ICTV) (Elliott et al. 2000). Official virus names recognized by the ICTV are italicized. Related distinct virus lineages possibly representing additional species, strains or subspecies are not italicized; nd, none documented.

Hantaviruses are generally named after the place they were first detected. The prototype virus for the Hantavirus genus, Hantaan virus, was isolated from the *Apodemus agrarius,* trapped near the Hantaan river (Lee et al. 1978). The natural hosts of Seoul virus (SEOV) are black and Norway rats (*Rattus rattus and R. norvegicus*) (Lee et al. 1982, Schmaljohn et al. 1985), which are much more mobile than hosts of other hantaviruses. Traveling in ships, rats are worldwide in distribution, as is the SEOV. PUUV, the etiologic agent of NE, was detected in the lungs of a bank vole (*Clethrionomys glareolus*) trapped in Puumala (Brummer-Korvenkontio et al. 1980), and isolated and adapted in a cell culture from lungs of a bank vole trapped in Sotkamo (Schmaljohn et al. 1985). *Clethrionomys glareolus* belongs to the *Arvicolinae* subfamily. For other Hantavirus species found in *Arvicolinae* hosts, for example Tula virus, Prospect Hill virus, Khabarovsk or Topografov virus, a pathogenecity for humans has not been shown, except in one case, when serological evidence for a previous Tula virus infection was obtained from the serum of a blood donor living near a Tula virus focus in Moravia, Czech Republic (Vapalahti et al. 1996b).

A decade ago, a further distinct hantavirus was isolated from the yellow-necked mouse (*A. flavicollis*) near Dobrava village in Slovenia (Avsic-Zupanc et al. 1992, Avsic-Zupanc et al. 1995), and subsequently a related virus from striped field mice (*A. agrarius*) on the island of Saaremaa, Estonia (Plyusnin et al. 1997b, Nemirov et al. 1999). Using the criteria currently selected to define the hantavirus species (Elliott et al. 2000), Dobrava virus (DOBV) and Saaremaa virus (SAAV) are considered distinct species (Plyusnin and Morzunov 2001, Brus Sjölander et al. 2002, Plyusnin 2002). The distribution of *A. flavicollis* and *A. agrarius* overlap over most Europe (Vapalahti et al. 2003). However, *A. agrarius* is not seen in westernmost Europe. Interestingly, in the regions where SAAV in *A. agrarius* has been suggested to be predominant (Estonia, Russia), the clinical outcome of HFRS seems to be milder than in cases associated with *A. flavicollis*-carried DOBV in the Balkans (Lundkvist et al. 1997a).

Sin Nombre virus (SNV) has been identified in deer mice (*Peromyscus maniculatus*) in southeast USA in 1993 (Nichol et al. 1993). Since then several new hantavirus species have been identified in mice in the Americas (Table 1).

Epidemiology

In Asia, HFRS is mainly caused by HTNV infections and found in rural areas (Lee and van der Groen 1989). Urban cases of HFRS in Asia have been reported to be caused by SEOV infection. In Europe, most hantavirus infections are caused by PUUV, followed by DOBV (Mustonen et al. 1998b, Vapalahti et al. 2003) and SAAV (Vapalahti et al. 2003). No clear evidence is available of SEOV-related HFRS in Europe, although SEOV has been seen in rats and antibodies in humans in Europe (Vapalahti et al. 2003). Along with its host, the bank vole, PUUV is reported throughout most of Europe with the exception of the Mediterranean coastal regions and most of the Iberian Peninsula and Greece and northernmost Norway (Mustonen et al. 1998b, Vapalahti et al. 2003). Most PUUV infections are reported from the European part of Russia and from Scandinavia, but numbers of cases also occur annually in Estonia, Latvia, Germany, Belgium, France, Austria, Bosnia-Herzegovina, the Netherlands, the Czech Republic, Slovakia, Slovenia and Croatia (Plyusnin et al. 2001, Markotic et al. 2002b, Vapalahti et al. 2003).

In Scandinavia, most PUUV infections are reported in Finland, where approximately 1,000 NE cases are serodiagnosed annually (Brummer-Korvenkontio et al. 1999). It has been calculated that possibly some 70% of PUUV infections still remain without serodiagnosis as judged by the seroprevalence, which is 5% in the Finnish population (Brummer-Korvenkontio et al. 1999). This is probably due to subclinical or mild disease, or limited use of serological verification for the diagnosis of NE. The diagnosis incidence is 19 per 100,000 for the whole country, this being apparently the highest figure recorded in the world. The highest incidences of NE cases, up to 80-90/100,000, have been measured in eastern Finland. In the Pirkanmaa region the incidence is 20/100,000 (Brummer-Korvenkontio et al. 1999). Besides PUUV, no other hantavirus has been observed to induce HFRS in Finland so far (Vapalahti, personal communication 2003). On average 100-300 and 50 serologically verified NE cases occur annually in Sweden and in Norway, respectively (Mustonen et al. 1998b, Vapalahti et al. 2003). In an endemic region in northern Sweden, 9% of the population has antibodies to PUUV (Ahlm et al. 1997).

In the European part of Russia the incidence of HFRS is about 5/100,000. PUUV is the most frequent cause of HFRS, and presumably SAAV-induced cases occur as well (Vapalahti et al. 2003). In the Far East, more severe HFRS is recognized, probably caused by HNTV. Both PUUV and SAAV have recently been found to be common in Estonia. The highest hantavirus seroprevalence rates were found on Saaremaa Island (SAAV) and in the central/south-western parts of Estonia (PUUV) (Golovljova et al. 2002). In Latvia, both SAAV and PUUV have been shown to be widely distributed (Lundkvist et al. 2002).

Hundreds of HFRS cases have been reported in the Balkans during the past few years, with a majority of cases due to PUUV infections (Lundkvist et al. 1997b), while DOBV-induced severe HFRS has been reported in Albania, Greece, Slovenia, Bosnia and Croatia (Antoniadis et al. 1996, Lundkvist et al. 1997a, Avsic-Zupanc et al. 1999, Markotic et al. 2002b).

HPS in North America has been associated mainly with SNV infections. There are also reports of New York-, Bayou virus- and Black Creek Canal virus-induced HPS cases. In South and Central America, HPS cases have been reported from Argentina, Brazil, Bolivia, Chile, Uruguay, Panama and Paraguay, and Andes virus seems to be the major pathogen (Enria et al. 2001).

In northern Europe, the bank vole exhibits pronounced 3-4 year population cycles thought to be caused by rodent predators (Plyusnin et al. 2001), and most human cases occur in the increase and peak phases of the cycle. In Finland and Sweden, it has been found that these cycles are not synchronous over the whole country, and epidemics are encountered in different areas during different years. In temperate Europe, the bank vole population is much more stable, basically seasonal, increasing in summers and declining in winters. However, rodent populations occasionally increase when heavy seed crops of oak and beech lead to improved survival. This is reflected in a rise in human epidemics of HFRS (Mustonen et al. 1998b).

In Finland most NE cases occur during the winter months. The risk of acquiring hantavirus infections is associated with activities and professions involving exposure to rodents and their excreta. These include farming (Groen et al. 1995b, Ahlm et al. 1998b, Vapalahti et al. 1999), forest work (Groen et al. 1995b, Ahlm et al. 1998b) and military field exercises (Niklasson et al. 1992).

Further, an increased seroprevalence has been found in European mammalogists with neutralizing antibodies predominantly directed against PUUV (Nuti et al. 1992, Zöller et al. 1995). The PUUV seroprevalence in Finnish rodent specialists is 50% (Vapalahti et al. 1995b).

In Sweden, Finland and Estonia, males have been found to suffer more often from serodiagnosed NE than females (male-to-female ratios 2.0:1, 1.8:1 and 1.8:1, respectively) (Niklasson et al. 1993, Brummer-Korvenkontio et al. 1999, Golovljova et al. 2002). The average age at contracting NE is 40 years in males and 44 years in females. Both for males and females, the seroprevalence appears to increase with advancing age (Niklasson et al. 1987, Ahlm et al. 1994a, Brummer-Korvenkontio et al. 1999), probably by reason of an increasing number of contacts with sources of infection over the years. Prevalence rates of NE are very low in children (Brummer-Korvenkontio et al. 1999), and children have been found to suffer from a clinically milder NE than adults (Ahlm et al. 1994b, Mustonen et al. 1994c).

Hantaviral clinical syndromes

Hemorrhagic fever with renal syndrome

As indicated by the name, the different forms of HFRS are febrile illnesses which generally involve hemostatic and renal disturbances. The clinical picture of HFRS is highly variable, from asymptomatic to fatal. HTNV- and DOBV-induced infections carry a considerable mortality, whereas diseases caused by PUUV, SAAV and SEOV are less severe. However, the differences in clinical severity within illnesses caused by one virus serotype may be just as great as between the different serotypes. In HTNV-induced HFRS, for example, 30% of patients run a mild clinical course, 50% a moderate, and only 20% a severe course (Lee and van der Groen 1989). The cause of this variation is unknown; it may involve the virulence of individual viral strains, the infective dose, or host

factors. The differences in the prevalence of clinical symptoms and findings in patients with different types of hantaviral diseases are shown in Table 2, and corresponding differences in the prevalence of laboratory findings are shown in Table 3.

Table 2. Clinical symptoms and findings in patients with hantaviral syndromes.

Symptom/finding	HFRS HNTV (Lee 1991)	SEOV (Lee 1991)	DOBV (Avsic-Zupanc et al. 1999)	NE PUUV (Lähdevirta 1971)	PUUV (Settergren et al. 1989)	PUUV (Mustonen et al. 1994a)	HPS SNV (Duchin et al. 1994)
Number of patients	104	40	19	76	74	126	17
Fever (%)	100	95	84	100	99	98	100
Nausea (%)	91	48	79 (vomiting)	78	84	58	71 (or vomiting)
Back pain (%)	91	80	84	82	82	54	29
Abdominal pain (%)	92	72	74	67	65	43	24
Headache (%)	97	45	63	90	85	62	71
Myalgia	75	51	47	na	69	27	100
Visual disturbances (%)	57	9	42	12	31	36	0
Petechiae (%)	94	43	na	12	1	2	0
Hemorrhagic manifestations	Minor 37%, major 34%	Minor 18%, major 5%	Macroscopic hemorrhagic complications 26%	Epistaxis 10.5%, macroscopic hematuria 2.6%, conjunctival bleedings 6%	Epistaxis 28%, macroscopic hematuria 1%, conjunctival bleedings 16%	10%	0
Diarrhea (%)	na	28	47	12	20	18	59
Cough (%)	31	13	na	6	32	14	71
Hypotension (%) (SBP<90 mmHg)	28	10	21 (shock)	2	1	11 (<100 mmHg)	50 (<100 mmHg)
Dialysis treatment (%)	40	20	47	1/76 (1%)	0	7/126 (6%)	0
Oliguria (%) (<400 ml/day)	60 (<500ml/d)	50	47	54	70	na	na
Polyuria (%) (>2000 ml/day)	95	91	na	97	97	na	na

Abbreviations: SBP = systolic blood pressure, na = not available.

Table 3. Laboratory findings in patients with hantaviral syndromes.

Finding	HFRS HNTV (Lee 1991)	SEOV (Lee 1991)	DOBV (Avsic-Zupanc et al. 1999)	NE PUUV (Lähdevirta 1971)	PUUV (Settergren et al. 1989)	PUUV (Mustonen et al. 1994a)	HPS SNV (Duchin et al. 1994)
Number of patients	104	40	19	76	74	126	17
B-$Leuk_{max}$ >10.0 x10^9/l (%)	91	75	53	57	23 (>12.0 x 10^9/l)	50 (> upper reference limit of the assay)	na
B-$Leuk_{max}$	na	na	18.3 (mean)	na	7.8 (median)	11.4 (mean)	10.4 (median)
S-$Crea_{max}$ > upper reference limit of the assay (%)	>150 µmol/l in 97%	>150 µmol/l in 73%	97	86	96	94	none had S-Crea >220 µmol/l
S-$Crea_{max}$ (µmol/l) (mean)	na	na	617	na	386	439	123 (median)
B-$Throm_{min}$ <100 x 10^9/l (%)	96 (<150 x 10^9/l)	80	68 (<150 x 10^9/l)	20	52	75 (< lower reference limit of the assay)	na
B-$Throm_{min}$ (10^9/l)	na	na	33 (mean)	na	96	117 (mean)	64 (median)
S-CRP_{max} (mg/l) (mean)	na	na	na	na	60	52	na
Proteinuria (%)	100	100	97	100	100	94	40
Proteinuria of nephrotic range (>3.5 g/l) (%)	na	na	na	na	na	25	na
Hematuria (%)	85	na	97	87	85	58	57
Hypoproteinemia/ hypoalbuminemia	S-Alb<30 g/l in 73%	na	na	S-Prot<60 g/l in 24%	S-Alb<36 g/l in 64%	S-Prot<30 g/l in 50%	median S-Alb_{min} 30 g/l

Abbreviations: S-$Crea_{max}$ = the highest serum creatinine concentration, B-$Leuk_{max}$ = the highest blood leukocyte count, B-$Throm_{min}$ = the lowest blood thrombocyte count measured during hospital care, na = not available.

In severe forms of HTNV-induced HFRS cases, five different phases are recognized: febrile, hypotensive, oliguric, polyuric and convalescent. After an incubation period which is generally 2-3 weeks, there is a sudden onset of disease with fever, chills, headache, myalgia, nausea, abdominal and/or back pains and tenderness of the renal area (Gajdusek 1982, Lee and van der Groen 1989). Blurred vision with myopia is a typical complaint. In addition, most patients have facial flushing. Hematuria and proteinuria appear as the first signs of renal involvement, whereas the glomerular filtration rate (GFR) is still unaffected. This febrile phase usually lasts for 3-7 days.

The hypotensive phase can last from hours to 2 days, and is often associated with hemoconcentration and normal or low serum protein concentrations, and with edema, often located in the retroperitoneum. Thrombocytopenia is a characteristic finding and may contribute to bleeding manifestations such as petechiae, epistaxis and in severe cases, fatal gastrointestinal or intracranial hemorrhages. The mechanism of thrombocytopenia is unresolved, but a defective endothelium probably plays a role in this, leading to increased consumption of thrombocytes (Cosgriff et al. 1991). In severe cases, clinical shock occurs and one third of HFRS deaths are associated with irreversible shock in this stage (Lee and van der Groen 1989).

In the oliguric phase (3-7 days), urinary output declines and sometimes anuria ensues. Weight gain and rise in blood pressure follow. One half of fatalities occur during this phase. Polyuria heralds recovery. Diuresis, due partly to impaired tubular function, may be profound. Finally, there is a convalescent phase which may be prolonged over weeks or months. In individual cases of HFRS, not all of these phases are always apparent and they may overlap to a considerable degree (Lee and van der Groen 1989).

The clinical picture of DOBV infection is consistent with that of HTNV-induced HFRS (Tables 2 and 3). PUUV and DOBV coexist in Croatia and Slovenia and are seen to induce HFRS with significant differences in severity (Avsic-Zupanc et al. 1999, Markotic et al. 2002b). DOBV-infected patients have more frequently suffered from acute renal failure requiring dialysis treatment, shock, visual disturbances, and severe thrombocytopenia, hemorrhagic complications and disseminated intravascular coagulation (DIC) than patients with PUUV (Avsic-Zupanc et al. 1999, Markotic et al. 2002b). In Asia, SEOV-caused urban cases occur. SEOV infection exhibits a shorter clinical course with milder renal dysfunction but more severe abdominal symptoms and hepatic involvement than those in HTNV infection (Lee 1991).

Nephropathia epidemica

In Finland, most PUUV infections remain without serological diagnosis due to subclinical or mild disease, or to the lack of use of serological verification for the diagnosis of NE (Brummer-Korvenkontio et al. 1999). It is thus obvious that only the most severe cases are hospitalized. The following description of NE patients is based only on hospital-treated patients, since no data are available on PUUV infections which are subclinical or treated in health-care centers.

Due to the relative mildness of NE, the five phases typically seen in HTNV-induced HFRS may be difficult to distinguish from each other, and one (or more) is usually absent. The incubation period varies from 2 to 4 weeks. The disease starts abruptly with high fever, chills and headache, followed by nausea and vomiting as well as abdominal and back pains (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a). Somnolence is common. Visual disturbances are often recorded, and a myopic shift is present in more than one-third of patients. This is due to a forward movement of the anterior diaphragm and thickening of the crystalline lens (Kontkanen et al. 1994).

Beginning in the late febrile phase, urinalysis discloses proteinuria and hematuria in almost all cases. Renal impairment follows, as indicated by oliguria and rising serum creatinine levels (Lähdevirta 1971). Leukocytosis is present in 23-57% of patients with acute NE (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a), and correlates with the severity of the renal disease as measured by serum creatinine (Mustonen et al. 1994b). A shift to the left is fairly common, and recently a case was reported with pronounced monocytosis (Ala-Houhala et al. 2000). Thrombocytopenia is encountered in a majority of patients, though bleeding manifestations are usually mild. Increased hematocrit is a common finding on admission, but later transient anemia follows. Other typical findings include a slight, transient increase in liver aminotransferases (Lähdevirta 1971, Settergren et al. 1989, Mustonen et al. 1994a). Electrolyte abnormalities are common, including hypocalcemia, hyperphosphatemia, hyponatremia and hypokalemia (Mustonen et al. 1994a).

Pulmonary involvement has been described in 16-53% of patients with NE. The radiological findings include parenchymal infiltrates, pleural effusions and

atelectasis (Lähdevirta 1971, Linderholm et al. 1992, Mustonen et al. 1994a, Kanerva et al. 1996). Pulmonary edema has been described in 4% of hospitalized patients (Kanerva et al. 1996). Central nervous system-related symptoms occur frequently in NE. In a Swedish study involving 26 patients with acute NE, the most common symptoms were headache, insomnia, vertigo and nausea (Ahlm et al. 1998a). Minor white-matter lesions were found in about half of the patients investigated by brain magnetic resonance imaging. Reports on hypophyseal hemorrhage and panhypopituitarism associated with NE are several (Forslund et al. 1992, Settergren et al. 1992, Valtonen et al. 1995, Hautala et al. 2002), and it is possible that hypopituitarism is more common than currently assumed.

Abnormalities in the electrocardiogram are reported in 20% of hospital-treated patients, including non-specific T-wave and ST-segment abnormalities. Regional left ventricular contraction abnormalities in echocardiography have been detected in 7 out of 20 patients during the acute phase of NE (Mustonen et al., abstract at the 3rd International Conference on HFRS and Hantaviruses 1995). PUUV infection may affect almost every organ, and some anecdotally reported complications seen in patients with NE are shown in Table 4.

In the differential diagnosis of NE other viral and bacterial infections, as well as other causes of acute interstitial nephritis and acute renal failure, must be taken into account.

Table 4. Rare complications of nephropathia epidemica

Complication	Reference
Acute disseminated encephalomyelitis	(Toivanen et al. 2002, Krause et al. 2003)
Cerebral haemorrhage	(Settergren et al. 1991b, Zeier et al. 1992, Alexeyev and Morozov 1995)
Meningoencephalitis	(Lähdevirta 1971, Launes and Hautanen 1988, Mustonen et al. 1994a, Bergmann et al. 2002)
Guillain-Barré syndrome	(Forslund et al. 1992, Esselink et al. 1994)
Pancreatitis	(Settergren et al. 1992)
Panhypopituitarism	(Forslund et al. 1992, Settergren et al. 1992, Valtonen et al. 1995, Hautala et al. 2002)
Perimyocarditis	(Mustonen et al. 1994a, Bergmann et al. 2002)
Urinary bladder paralysis	(Alexeyev and Morozov 1995)
DIC syndrome	(Settergren et al. 1989, Linderholm et al. 1991, Clement et al. 1994)

Renal involvement

Renal involvement in HFRS results in transient proteinuria, microscopic hematuria and acute renal failure with oliguria followed by polyuria and spontaneous recovery. The need for dialysis treatment varies from 6% in hospitalized patients with NE (Mustonen et al. 1994a) to 47% in patients with DOBV-induced HFRS (Avsic-Zupanc et al. 1999). It should be noted that creatinine concentrations can be quite normal even in hospital-treated patients (Settergren et al. 1989, Settergren et al. 1990, Mustonen et al. 1994a). Polyuria may be seen without any preceding oliguria, reflecting tubular dysfunction (Lähdevirta 1971, Settergren et al. 1990, Lee 1991, Mustonen et al. 1994a).

The characteristic histopathological finding in renal biopsy during the acute phase of NE is acute tubulointerstitial nephritis (Figure 1), and infiltrating cells include lymphocytes, plasma cells, monocytes, macrophages and polymorphonuclear leukocytes (Collan et al. 1991, Mustonen et al. 1994b, Temonen et al. 1996). Medullary hemorrhages are found in 20-60% of acute-phase biopsies (Figure 1) (Collan et al. 1991, Mustonen et al. 1994b). Tubular epithelial flattening, vacuolization in tubular epithelial cells, luminal casts and distended lumina, and occasional tubular cell necrosis and mitoses are found. Deposits of immunoglobulin G (IgG), IgM, complement factor (C) 3 and fibrinogen are observed in about half of the acute-phase NE kidneys along the tubular basement membrane by immunofluorescence staining (Collan et al. 1991). Electron microscopy in NE kidneys shows focal collapsing and thickening of the tubular basement membrane (Collan et al. 1991).

Despite common and sometimes massive proteinuria seen in patients with NE, histological glomerular changes are surprisingly mild, and reported only in one fourth of cases (Mustonen et al. 1994b). They include slight mononuclear hypercellularity and minor mesangial proliferation (Collan et al. 1991, Mustonen et al. 1994b). Immunofluorescence studies have revealed occasional glomerular deposits of IgA, IgG, IgM, C3 and C1q, but these have not been related to any other histological findings (Collan et al. 1991, Mustonen et al. 1994b). In electron microscopy, only slight degenerative changes have been seen in the glomerular endothelial cells (Collan et al. 1991).

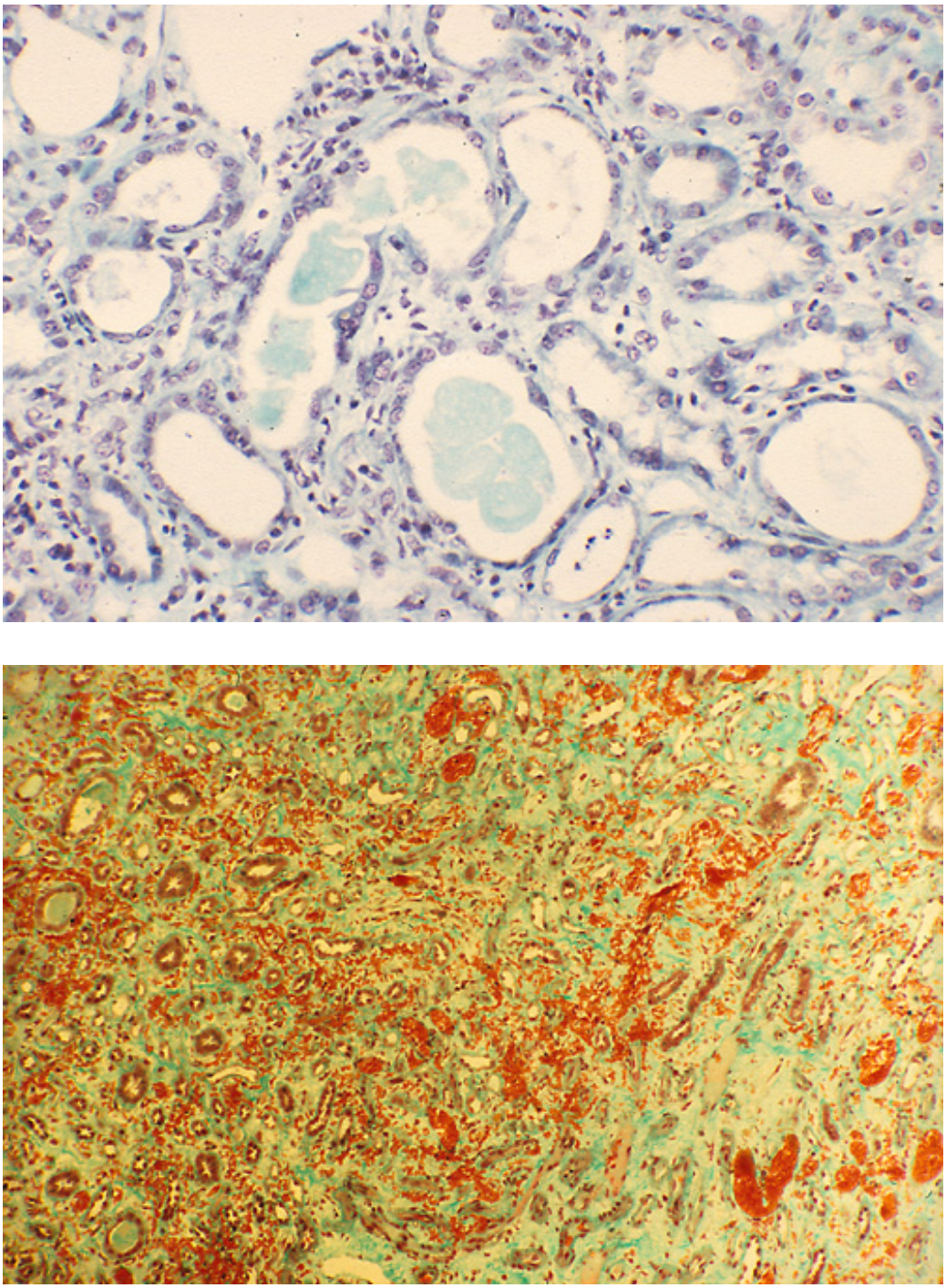

Figure 1. A renal biopsy sample obtained during the acute phase of NE reveal interstitial edema and an inflammatory cell infiltrate associated with tubular epithelial flattening, vacuolization in tubular epithelial cells, luminal casts and distended lumina, and tubular cell necrosis (above) (hematoxylin and eosin staining). Hemorrhage in corticomedullary junction (below) (Masson's trichrome staining). The pictures are provided by Dos. Heikki Helin, Tampere University Hospital, Finland.

Proteinuria in HFRS begins abruptly and can be massive. Nephrotic-range proteinuria (>3.5 g/day) has been reported in 25% of hospital-treated patients with NE (Mustonen et al. 1994a). Proteinuria is non-selective (Cosgriff 1991), indicating a defective glomerular barrier. Recently, an increased glomerular permeability associated with impairment of both the size- and charge-selectivity properties of the glomerular filter was shown in NE patients (Ala-Houhala et al. 2002). Concomitant urinary loss of low-molecular-weight proteins such as β_2-microglobulin (Settergren et al. 1990, Cosgriff 1991) and α_1-microglobulin (Ala-Houhala et al. 2002) indicates that tubular injury also contributes to the proteinuria. No relation has been found between the severity of histological changes and protein excretion in NE (Mustonen et al. 1994b). Proteinuria rapidly decreases in the polyuric phase. The occurrence of microscopic hematuria is almost the rule, but macroscopic hematuria is rare (Lähdevirta 1971, Settergren et al. 1990, Mustonen et al. 1994a, Settergren et al. 1997). Leukocyturia is present in 28% and glucosuria in 9% of NE patients (Mustonen et al. 1994a). Urine microscopy has revealed granular or hyaline casts in 25% of the patients (Settergren et al. 1990).

The GFR studied either by ^{51}CrEDTA or inulin clearance, and effective renal plasma flow (ERPF) as studied by para-aminohippurate clearance have been shown to be significantly reduced in patients during the acute phase of NE (Settergren et al. 1990, Ala-Houhala et al. 2002). Renal involvement in the acute phase is further characterized by a high filtration fraction (FF) (Ala-Houhala et al. 2002). The fall in systemic blood pressure does not explain these functional changes, since renal failure may occur without hypotension and blood pressure levels do not correlate with the severity of renal failure in HFRS. Probably intrarenal events play an important role in the development of renal failure in HFRS (Cosgriff 1991).

Renal ultrasound changes are common during the acute phase of NE. In a recent study of 23 hospital-treated patients renal length was shown to be increased in all patients in the acute phase of NE (Paakkala et al. 2002). The resistive index was abnormal in 12 and fluid collections (pleural, perirenal, pericardial, ascites) were found in 13 patients. The severity of ultrasound

findings was associated with fluid overload and degree of clinical renal insufficiency.

Hantavirus pulmonary syndrome

The original description of HPS came from a study of SNV-induced infections (Duchin et al. 1994). SNV is a major agent responsible for HPS in North America (Duchin et al. 1994, Simpson 1998, Enria et al. 2001, Peters and Khan 2002). Instead of hemorrhages and renal failure, HPS is typically characterized by severe pulmonary involvement (Duchin et al. 1994, Chapman et al. 2002) but the infection can also present without respiratory failure (Kitsutani et al. 1999). The onset of symptoms occurs at a median of 14-17 days after exposure (Simpson 1998, Enria et al. 2001, Peters and Khan 2002). The clinical features of HPS were originally divided into four phases (febrile, cardiopulmonary, diuretic and convalescent), in contrast to the five for HFRS.

The prodromal symptoms include fever, myalgia and malaise. Also headache, anorexia, nausea, vomiting, diarrhea, back and abdominal pains may occur (Chapman et al. 2002). The prodrome typically lasts 3-5 days (Peters and Khan 2002). The next stage of disease is characterized by shock and pulmonary edema, which may progress rapidly (in 4-24 hours). Patients often have a cough, tachypnea, tachycardia and hypotension (Peters and Khan 2002). Pulmonary edema is non-cardiogenic in origin, and pulmonary capillary wedge pressures are normal, as is the heart size on the X-ray. Chest radiographic findings were recently studied in Canadian patients with HPS (Boroja et al. 2002). Bilateral parenchymal or bilateral interstitial and alveolar infiltrates with pleural effusions were noted. The radiographic findings were associated with the severity of disease. Once pulmonary edema is present, the disease proceeds rapidly, with patients dying within 24-48 hours, hypoxia, circulatory collapse or both being the immediate cause of death. The circulatory collapse differs from the findings seen in septic shock, and the most commonly registered pattern is low cardiac output, high systemic vascular resistance and hypotension. The case fatality has been decreasing from more than 50% in 1993 to 20% in 1997 (Enria et al. 2001).

As shown in Table 3, the occurrence of leukocytosis, thrombocytopenia and elevated hematocrit are typical signs of an infection by SNV or related hantaviruses (Duchin et al. 1994, Nolte et al. 1995, Zaki et al. 1995, Chapman et al. 2002). The kidney is not usually affected in a major way as in HFRS, and elevated serum creatinine concentrations are thought to be related to shock and hypovolemia. Proteinuria is, however, a common finding (Duchin et al. 1994, Nolte et al. 1995, Zaki et al. 1995). The diuretic phase is characterized by a rapid clinical improvement. The convalescent phase may last up to 2 months with patients recovering apparently fully (Enria et al. 2001). The long-term prognosis of survivors after HPS has not been studied.

There are probably also subclinical and/or mild HPS cases, since high human seroprevalence rates of antibodies against hantaviruses have been reported in Paraguay and northern Argentina, ranging from 7 to 21% among asymptomatic groups and community residents (Williams et al. 1997).

Comparison of the syndromes

HPS is characterized by severe cardiopulmonary dysfunction and shares many similarities to HFRS, including fever, gastrointestinal symptoms, hypotension, hemoconcentration, thrombocytopenia and leukocytosis. Pulmonary involvement is common also in patients with acute NE (Linderholm et al. 1992, Kanerva et al. 1996). Occasionally, in severe NE cases, respiratory failure has mimicked that in HPS (Clement et al. 1994, Kanerva et al. 1996, Takala et al. 2000).

In turn, there are several reports on renal involvement in HPS. Proteinuria has been observed in 40% and hematuria in 57% of SNV-induced HPS cases (Duchin et al. 1994). Renal involvement seems to be more common in Black Creek Canal virus- and Bayou virus-induced HPS than in SNV-infections (Khan et al. 1995, Rollin et al. 1995, Hjelle et al. 1996, Khan et al. 1996), and is especially common in Andes virus-induced disease in South-America (Lazaro et al. 2000). A systematic review of 25 Andes virus-induced HPS cases from Argentina documented acute renal insufficiency in a majority of patients, serum creatinine being higher than 176 μmol/l in 53% (Lazaro et al. 2000). Two

patients required hemodialysis treatment. Urinalysis was abnormal in all 12 cases studied with presence of protein, red blood cells and granular casts.

It should be also noted that there is no absolute assignment of renal disease and HFRS on the one hand, and lung disease and HPS on the other. It seems that HFRS and HPS do not truly represent two clinically distinct diseases, rather a continuum from renal disease in HFRS to pulmonary disease in HPS. This is most likely a result of the fact that hantaviruses infect and replicate primarily in endothelial cells, which are widely distributed.

Laboratory diagnosis

The laboratory diagnosis of HFRS and HPS is based on serology using principally IFA or highly sensitive and specific enzyme-linked immunosorbent assays (ELISA). Also reverse transcription-polymerase chain reaction (PCR)-assays have been successfully used. However, the viremia in HFRS patients is short-termed, with only 67% of acute-phase PUUV-infected patients (Hörling et al. 1995, Plyusnin et al. 1997a, Plyusnin et al. 1999) and about 40% of acute-phase DOBV-infected patients (Papa et al. 1998) being positive for viral RNA in the reverse transcription-PCR currently used. Hence the diagnostic value of PCR assays is limited. Furthermore, virus isolation is seldom successful in hantaviral infections.

The first serological method used for diagnosis was detection of hantavirus-specific antibodies in patients with HTNV-induced HFRS by IFA using lung tissue of *A. agrarius* as antigen (Lee and Lee 1976). Subsequently, IFA with cell culture-propagated viral antigens came to be used in clinical laboratories (Brummer-Korvenkontio et al. 1980, Hedman et al. 1991, Settergren et al. 1991a, Settergren et al. 1997). A modified IFA based on the avidity of PUUV-specific IgG was developed to distinguish acute-phase disease from old immunity (Hedman et al. 1991).

ELISAs employ either native or recombinant N proteins as diagnostic antigens (Lundkvist et al. 1993b, Vapalahti et al. 1996a, Brus Sjölander et al. 1997). Application of complete N protein for serodiagnosis is optimal since N

protein has been demonstrated to be the major antigen in the human antibody response (Lundkvist et al. 1993a, Lundkvist et al. 1993b, Vapalahti et al. 1995a), and more sensitive compared to its terminal fragments (Brus Sjölander et al. 1997, Kallio-Kokko et al. 2000). During recent years, a number of recombinant hantavirus antigens have been produced (Vapalahti et al. 1996a, Brus Sjölander et al. 2000, Kallio-Kokko et al. 2000).

The optimal methodology for a rapid serological diagnosis of acute hantavirus infection is based on the detection of virus-specific IgM, since in endemic areas there is a high prevalence of virus-specific IgG due to previous infections. Furthermore, the majority of hantavirus-infected patients present IgM antibodies already at the time of onset of disease (Groen et al. 1992, LeDuc et al. 1990, Lundkvist et al. 1993b). In rare cases (<2%), PUUV-specific IgM antibodies may remain negative for up to 5 days after the onset of symptoms (Kallio-Kokko et al. 1998). Also the IgG response is rapid: 85% of patients are IgG-seropositive by the 3rd day and 100% by the 6th day (Hedman et al. 1991). Follow-up sera should be requested if primary samples were drawn early.

A new point-of-care immunochromatographic PUUV-specific IgM test has been developed (Hujakka et al. 2001). The analytical sensitivity and specificity of this rapid test have been shown to be 100 and 99%, respectively, for unfrozen serum samples when ELISA (Vapalahti et al. 1996a, Kallio-Kokko et al. 1998) for IgM and an IFA (Hedman et al. 1991) for IgG antibodies were used as reference methods.

The serological cross-reactivity among the different hantavirus serotypes is pronounced in assays such as IFA or ELISA, and to date the only reliable test for distinguishing the antibody responses against different closely related hantaviruses is the focus-reduction neutralizing test or the plaque reduction neutralizing test (Lundkvist et al. 1997b).

Treatment

The treatment of patients with HFRS relies on good supportive care, including management of electrolyte, fluid and acid-base homeostasis and pain relief. The

therapy must be carefully monitored according to the patient´s fluid status, amount of diuresis and kidney function. Intensive care facilities and ventilation support are commonly needed in HPS and sometimes also in severe HFRS cases. Treatment of acute renal failure includes dialysis treatment when needed.

The only medication to have shown some benefit in the treatment of hantaviral diseases is the antiviral drug ribavirin. A controlled clinical trial of ribavirin in HTNV-induced HFRS patients in China revealed a significant reduction in fatality and severity of symptoms when administered early after onset of disease (Huggins et al. 1991). In contrast, a recent study on HPS patients revealed no appreciable drug effect (Chapman et al. 1999).

Prevention

Prevention of exposure to rodents and their excreta reduces the risk of hantaviral infection. Efforts should include the elimination of rodent food sources inside and around homes, and avoidance of situations where contaminated dust can be inhaled (Kruger et al. 2001). These measures can slightly reduce the individual risk of infection. However, for a more general prevention of human infections, especially in occupational risk groups, the development of vaccines is necessary.

Several vaccines based on inactivated hantaviruses (HTNV, SEOV) have been generated, and a few of them are also commercially produced and licensed for human use. Since the introduction of Hantavax™ in 1990, the total number of hospitalized HFRS cases in South Korea decreased from 1234 cases in 1991 to 687 cases in 1996 (Kruger et al. 2001). Recently, vaccine efforts have sought to elicit protective immune responses by using recombinant deoxyribonucleic acid (DNA) approaches. In the future, it might be possible to develop a recombinant DNA vaccine containing a combination of immunogens protective against all hantaviruses which cause human disease.

Pathogenesis of hantaviral infections

Vascular endothelium

Increased capillary permeability is characteristic of various types of hantavirus infections, and is thought to be of fundamental pathophysiological importance. Vascular leakage may explain many symptoms and features of human hantavirus infections, for example hypotension and abdominal pain due to retroperitoneal edema in HFRS, as well as the extravasations of fluid to alveolar space and pulmonary edema occurring in HPS (Cosgriff 1991, Kanerva et al. 1998a).

Vascular endothelial cells are postulated to be the main target of hantaviruses *in vivo.* Viral antigen has been detected in the endothelial cells of capillaries in many organs, including the brain, lungs, heart, spleen, liver and kidney in fatal HFRS (Kanerva et al. 1998a) and HPS cases (Zaki et al. 1995). Recently a hemorrhagic hypophysis positive for PUUV antigen in both neuroendocrine stromal and vascular endothelial cells was revealed in a fatal NE case (Hautala et al. 2002). Both pathogenic and non-pathogenic hantaviruses replicate predominantly in vascular endothelial cells and macrophages, but there is little if any damage to the infected endothelium (Yanagihara and Silverman 1990, Pensiero et al. 1992, Temonen et al. 1993, Zaki et al. 1995).

Importantly, Gavrilovskaya and associates (1998, 1999) have recently shown that pathogenic and non-pathogenic hantaviruses use β_3 and β_1 integrins, respectively, to enter endothelial cells. Integrins are heterodimeric receptors mediating cell-cell adhesion, cell migration and extracellular matrix protein recognition. Integrin-directed cellular migration is an essential function of endothelial cells in wound repair, angiogenesis and the maintenance of vascular integrity (Huttenlocher et al. 1996). β_3 integrins are abundant surface receptors in endothelial cells and platelets, play central roles in maintaining capillary integrity and have recently been found to bind receptors which regulate vascular permeability (Huttenlocher et al. 1996, Hodivala-Dilke et al. 1999). In fact, β_3 integrin knockout mice evince striking vascular permeability defects which result

in mucocutaneous hemorrhages and vascular permeability effects similar to those occurring during HFRS (Hodivala-Dilke et al. 1999).

Recently it was demonstrated that pathogenic hantaviruses (New York-1 virus, SNV, HTNV, PUUV and SEOV) inhibited integrin-directed endothelial cell migration, and that only migration on β_3, but not β_1, integrin ligands was inhibited by HPS- and HFRS-causing viruses (Gavrilovskaya et al. 2002). Thus, the ability of endothelial cells to migrate on integrin ligands was selectively inhibited only by pathogenic hantaviruses. Since β_3 integrins are linked to changes in vascular permeability and the maintenance of vascular integrity, these findings suggest a means by which hantavirus usage and regulation of β_3 integrins may contribute to the pathogenesis of hantaviral disease (Gavrilovskaya et al. 2002).

Further, it has been shown that hantaviruses are able to infect immature dendritic cells *in vitro* and induce their maturation and tumor necrosis factor α (TNFα) production while maintaining the ability to activate T-cells (Raftery et al. 2002). This suggests that hantavirus-infected migrating dendritic cells could provide a vehicle for dissemination of hantavirus from lung to lymph nodes and throughout the body.

Animal models

Although rodent models have been extensively explored in hantavirus research, they do not allow studies mimicking the pathogenesis of hantavirus infections in humans. Rodent models have nonetheless proved valuable for vaccine candidate studies (Schmaljohn et al. 1990, Lundkvist et al. 1996).

The lack of an adequate animal model has been a major obstacle in studying the pathogenesis of hantaviral diseases. In 1995, Groen and associates (Groen et al. 1995a) reported a study of PUUV infection of cynomolgus macaques. Although most of the monkeys became antibody-positive, no clinical signs which mirrored human hantavirus infection were observed. Recently, a successful infection of cynomolgus macaques by wild-type PUUV was reported (Klingström et al. 2002). The animals developed antibody responses similar to

what has been described for human patients, including rapidly increasing levels of neutralizing antibodies. The monkeys exhibited typical clinical signs of NE, including lethargy, anorexia, proteinuria, and/or hematuria, in addition to cytokine (interleukin (IL)-6, IL-10, and TNFα), CRP, creatinine and nitric oxide responses (Klingström et al. 2002).

Cynomolgus macaques have also been exposed to HPS-causing Andes virus (McElroy et al. 2002). The monkeys did not manifest clinical disease but developed antibodies against the viral nucleocapsid protein and a neutralizing antibody response. Andes virus has, however, been highly lethal in Syrian hamsters (Hooper et al. 2001). The characteristics of the disease in hamsters closely resembled that of HPS in humans.

Autopsy findings in human diseases

Lacking suitable animal models, much of the present knowledge regarding the pathophysiology of hantaviral diseases has been gained by autopsy and histopathological studies. Postmortem studies of HFRS patients revealed diffuse hemorrhages in the right atrium of the heart, hemorrhage and necrosis in the anterior lobe of the pituitary gland and pulmonary edema (Gajdusek 1982). In patients dying in the hypotensive phase of the disease, retroperitoneal edema and effusions of the body cavities were observed. The kidneys were enlarged with a pale cortex and a congested, hemorrhagic medulla (Gajdusek 1982). In few fatal cases of NE, autopsies have revealed hemorrhage and necrotic areas of the pituitary glands, venous congestion and hemorrhage of the kidneys as well as pulmonary edema (Valtonen et al. 1995).

Autopsy findings in HPS typically showed striking pulmonary edema and large pleural effusions, while retroperitoneal edema was typically absent (Zaki et al. 1995). Despite demonstrable SNV RNA in the vascular endothelium of many organs, morphological changes in the endothelium were uncommon.

B-cells

Human hantavirus infections initially induce high levels of virus-specific IgM (Groen et al. 1992, Lundkvist et al. 1993b, Elgh et al. 1998, Bharadwaj et al. 2000, Bostik et al. 2000). The response is directed mainly to the N and G2 proteins, while lower reactivity is detected against the G1 protein (Lundkvist et al. 1993b). This IgM response remains detectable during the convalescent phase, but is usually undetectable at three months post infection (Lundkvist et al. 1993b, Elgh et al. 1998). The IgG response arises later than the IgM response, and is mainly directed to the N protein (Groen et al. 1992, Lundkvist et al. 1993b, Elgh et al. 1998). IgG responses against the glycoproteins appear later during the convalescent phase, but have been shown to remain elevated for more than 10 years (Lundkvist et al. 1993b). Hantavirus-specific IgA responses seem to follow the kinetics of the IgM response in that they arise early (Elgh et al. 1998, Bharadwaj et al. 2000, Bostik et al. 2000, de Carvalho Nicacio et al. 2000). However, serum IgA seems to persist longer than IgM, as low levels of PUUV-specific IgA have been found in late convalescent sera (de Carvalho Nicacio et al. 2000).

Hantavirus infection is thought to leave a life-long immunity, and PUUV-specific anti-N protein IgG, as well as virus-neutralizing antibodies directed against G1 and G2, has been detected several decades after infection (Settergren et al. 1991a, Lundkvist et al. 1993b). Whether a previous PUUV infection protects from DOBV or SAAV infection or vice versa is not known. However, no cases of a second hantavirus infection in a preimmune person have ever been reported (Plyusnin et al. 2001).

The neutralizing antibody response develops early and is usually already present at the onset of disease (Hörling et al. 1992, Lundkvist et al. 1993b, Lundkvist et al. 1997b). As only low levels of glycoprotein-specific IgG are present in the early stages of infection, the neutralization has been attributed to the initial IgM and IgA responses (Lundkvist et al. 1993b, de Carvalho Nicacio et al. 2000). In contrast, late convalescent neutralizing IgG responses become highly serotype-specific (Lundkvist et al. 1993b, Lundkvist et al. 1997b). Neutralizing antibodies play an important role in the control of hantavirus

infections (Arikawa et al. 1992), and the severity of disease has been reported to correlate with neutralizing antibody titers in SNV-induced HPS, with lower neutralizing antibody titers in severe than in mild cases (Bharadwaj et al. 2000).

The humoral cross-reactivity between the different hantavirus serotypes is high (Lundkvist et al. 1997b). This is particularly pronounced within the group of viruses carried by *Murinae* rodents (HTNV, SEOV, DOBV), *Arvicolinae* rodents (PUUV, Prospect Hill virus, Tula virus, Topografov virus), and *Sigmodontinae* rodents (SNV, Andes virus), this due to the high level N protein homologies within these groups.

T-cells

Cellular immune responses have been held to be involved in the pathogenesis of hantavirus disease, as hantaviruses infect cells without causing any direct cytopathic effects. On the other hand, cellular immune responses also appear to be important in protection against (Yoshimatsu et al. 1996) and clearance (Asada et al. 1987) of hantavirus infections, as observed in animal studies.

An increased number of activated circulating CD8+ T cells (cytotoxic T lymphocytes, CTLs) with a decreased CD4:CD8 ratio (CD4+, T helper cells) has been described in HTNV-induced HFRS, along with an increased number of activated T-cells producing interferon-γ (IFN-γ) (Huang et al. 1994). In kidney biopsies obtained during the acute phase of NE, CD8+ T-cells dominated the lymphocytic infiltrate, while minor numbers of CD4+ T-cells and B-cells were detected (Temonen et al. 1996). In lung samples from lethal HPS cases, cell infiltrates were mainly composed of CD8+ T-lymphocytes and macrophages/monocytes (Zaki et al. 1995). Bronchoalveolar lavage fluid from NE patients has been shown to contain significantly more lysozyme-positive macrophages, CD8+ T cells and natural killer cells than that in healthy individuals (Linderholm et al. 1993). The findings may indicate the presence of a local host response to PUUV infection in the lower respiratory tract of patients with NE.

Futhermore, CTL epitopes have been identified in the N and G1 proteins from acute-phase HPS patients infected with SNV (Ennis et al. 1997), and

analysis of human memory CTL responses against laboratory-acquired HTNV infection have identified both virus-specific and cross-reactive CTL epitopes in the N protein (Van Epps et al. 1999). More recently, memory CTL responses were studied in Finnish individuals who had had NE 6-15 years earlier (Van Epps et al. 2002). Novel CD8+ CTL epitopes on the PUUV N protein were defined, and memory CTLs specific for these epitopes were found to be present at a high frequency in PUUV-immune individuals.

Complement

Viruses can activate the complement system either directly (alternative route) or via the antibody-related system (classical route). In viral diseases activation of classical route factors C2 and C4 has been shown. In HTNV-induced HFRS the peak complement activation has been observed to appear during the hypotensive stage and gradually disappear in the convalescent stage (Wang et al. 1986). Complement activation is also common in NE. In a study with 25 hospital-treated acutely ill NE patients, complement activation was observed in 23 out of 25 cases. Although both pathways were shown to be activated, the classical pathway activation with immune complexes or directly by viral components was associated with a severe clinical course of NE (Paakkala et al. 2000).

Cytokines

Cytokines are a group of small soluble or cell-membrane bound protein or glycoprotein messenger molecules which convey information between cells. Cytokines are generally divided into subgroups of interleukins, growth factors, chemokines, interferons and colony-stimulating factors. The key cytokines participating in the regulation of the inflammatory response are TNFα, IL-1, IL-6, IL-10 and IL-1 receptor antagonist (IL-1Ra). Functionally these cytokines can be divided into proinflammatory (IL-1, IL-6 and TNFα) and anti-inflammatory (IL-1Ra and IL-10) molecules.

TNFα is produced by monocytes, macrophages and T cells (Papadakis and Targan 2000). Despite its beneficial role in host defense, several pathological conditions have been associated with inappropriate TNFα production. High serum levels of TNFα lead to a number of signs and symptoms similar to those seen in NE, for example shock and tissue damage, vascular leakage, adult respiratory distress syndrome, acute tubular necrosis, DIC, thrombocytopenia and fever (Papadakis and Targan 2000).

IL-1 is a pleiotropic cytokine with two forms, IL-1α and IL-1β. Both are produced by monocytes and macrophages, and share a similar profile of functions (Dinarello 1996). IL-1 binds to cell surface receptors and activates B- and T-cell functions, regulates fever reaction, and participates in the initiation of acute-phase protein synthesis (Dinarello 1996). Further, IL-1 and TNFα have been shown to act synergistically in the induction of shock in animal models (Okusawa et al. 1988). IL-1α is largely found in cell cytosol, while IL-1β is the main secreted form of IL-1. IL-1 has receptors which are responsible for mediating the biological responses of IL-1 to target cells (Dinarello 1996).

The IL-1Ra, an endogenous inhibitor of IL-1α and IL-1β, is produced in many cells in response to the same stimulus as IL-1. IL-1Ra is able to inhibit IL-1-induced T-cell proliferation and activation of B-cells. Although IL-1Ra has a high binding affinity to the IL-1 receptor, 10-100-fold molar excesses are needed to inhibit IL-1 activity (Dinarello 1996). The principal sources of IL-1Ra are monocytes, macrophages and neutrophils. There is some evidence that the balance between IL-1 and IL-1Ra is important in the regulation of inflammatory responses.

Together with IL-1β and TNFα, IL-6 is one of the main proinflammatory cytokines. Its main functions appear to be induction of B-cell proliferation and differentiation, and regulation of production of the acute-phase proteins. It also possesses anti-inflammatory and endocrine functions (Papanicolaou et al. 1998). IL-6 is produced by various cell types, including fibroblasts, activated T and B cells and activated macrophages, as well as endothelial, mesangial and renal tubular cells. Administration to humans result in fever, fatigue, anorexia and influenza-like symptoms, anemia, leukocytosis, thrombocytosis and elevation of

acute-phase proteins (Papanicolaou et al. 1998). IL-6 effects are mediated by the IL-6 receptor.

Pathogenetic implications

There is a growing body of evidence that cytokines play a role in the pathogenesis of hantaviral diseases. In a Swedish study, high plasma levels of TNFα, soluble TNF receptors, IL-10 and IL-6 were detected in patients with acute NE (Linderholm et al. 1996). Interestingly, plasma concentrations of TNFα were shown to correlate directly with the serum creatinine level and inversely with blood pressure. Markers of systemic inflammation (neutrophil and monocyte CD11b expression, serum IL-6, IL-8, soluble IL-2 receptor and soluble E-selectin) have recently been studied in 19 patients with acute NE (Takala et al. 2000). These markers were higher in patients with NE than in healthy subjects. The findings correlated inversely with mean arterial pressure (soluble IL-2 receptor and monocyte CD11b expression) and minimum platelet count (soluble IL-2 receptor, IL-6, neutrophil and monocyte CD11b expression), but not with serum creatinine levels, suggesting that the severity of inflammation is related to hypotension and platelet consumption but not to renal injury.

Furthermore, Markotic and co-workers (2002a) have described a Croatian patient who presented with mild flu-like symptoms two weeks before the onset of NE. During the incubation phase, serum IL-6 concentrations were normal but increased during the acute phase of NE (Markotic et al. 2002a). IL-6 concentrations were still elevated on the 6th day after HFRS onset. Serum TNFα levels were not studied.

Several cytokines have also been detected in tissues from HFRS and HPS patients (Temonen et al. 1996, Mori et al. 1999). In kidney biopsies taken during acute NE immunofluorescence staining has revealed an increased expression of TNFα together with transforming growth factor β (TGFβ) and platelet-derived growth factor (PDGF) in the peritubular area of the distal nephron, at the same locations with the inflammatory cell infiltrate (Temonen et al. 1996). TNFα is known to increase vascular permeability and to increase the expression of

endothelial adhesion molecules (Tracey and Cerami 1994). Increased expression of intercellular and vascular cell adhesion molecules has been described in the interstitial and tubular space of NE kidneys, respectively (Temonen et al. 1996).

Immunohistochemical staining has revealed high numbers of cytokine-producing cells in the lung and spleen tissues obtained from subjects with fatal HPS but only low numbers in the livers and kidneys, suggesting that local cytokine production may play an important role in the pathogenesis of HPS (Mori et al. 1999). In contrast, one study revealed no change in the production of TNFα by alveolar macrophages after infection with SNV (Khaiboullina et al. 2000). Another recent study has shown that infection of cynomolgus macaques by wild-type PUUV results in increased serum levels of TNFα, IL-6 and IL-10 (Klingström et al. 2002).

In a panel of sera obtained from HFRS patients from the Korean epidemic during early 1950s, increased levels of IFN-γ, an important stimulator of cellular immune responses, were detected (Krakauer et al. 1994). The raised IFN-γ levels were shown to correlate to the severity of NE-like disease in macaques (Klingström et al. 2002), while low or no IFN-γ was reported in NE patients during the acute phase (Linderholm et al. 1996). The antiviral interferon IFN-α has been detected in both HFRS patients' sera (Krakauer et al. 1994) and in sera from PUUV infected macaques, in which IFN-α detection was associated with milder disease symptoms (Klingström et al. 2002). The observed cytokine patterns in patients and experimentally infected monkeys suggest that both inflammatory and cellular immune responses are activated during hantaviral disease.

Prognosis of hantaviral infections

Mortality

Mortality rates associated with hantavirus diseases are shown in Table 5. The mortality due to NE is very low (Brummer-Korvenkontio et al. 1999). However, some lethal cases have been described in Finland and Sweden (Linderholm et al. 1991, Forslund et al. 1992, Valtonen et al. 1995, Hautala et al. 2002), the deaths being mainly attributable to irreversible circulatory collapse and brain edema. In three out of four fatal NE cases, autopsies showed hemorrhage and necrosis of the pituitary gland (Valtonen et al. 1995).

Table 5. Mortality in hantaviral clinical syndromes.

Hantavirus	Hantaviral syndrome	Mortality	Reference
Hantaan	HFRS	2-7%	(Lee and van der Groen 1989)
Seoul	HFRS	About 1%	(Lee 1991)
Dobrava	HFRS	16%	(Avsic-Zupanc et al. 1999)
Puumala	NE	<0.1%	(Brummer-Korvenkontio et al. 1999)
Saaremaa	HFRS	To date no fatal cases described	(Plyusnin and Morzunov 2001, Golovljova et al. 2002)
Sin Nombre	HPS	20-50%	(Enria et al. 2001)

Sequelae

The prognosis of HFRS is held to be favorable. A study made by Lähdevirta (1971) revealed that none of the 76 patients who were treated for acute NE during the years 1957-66, was readmitted at any later date (up to 1966) due to a disease which could reasonably be connected to NE. Twenty patients were examined 1-6.5 years after NE, and none had proteinuria in urinalysis. Slightly reduced creatinine clearance values (below 100 ml/min/1.73 m^2) were detected in 5 out of 20 cases, with the lowest recorded value of 78 ml/min/1.73 m^2. Renal biopsy was performed in 3 cases and none was considered to be fully normal. Slight interstitial fibrosis and minimal glomerular changes were found, but regarded as inactive scars. The renal concentration capacity was evaluated from

the specific gravity of urine after 18 hours water fasting. Decreased values were found in 8 out of 20 patients. Blood pressure values over 140/90 mmHg were observed in 4 patients (Lähdevirta 1971). In a later study of 9 patients carried out 4-5 years after acute disease, glomerular function was found to be normal, but there were slightly abnormal values in tubular function tests (acidification and concentration capacity) in 5 patients (Lähdevirta et al. 1978). Mild nonspecific changes were seen in renal biopsies, possibly connected with the tubular dysfunction.

In a Greek study, twelve patients were examined 1-5 years after acute hantavirus infection and three with normal creatinine clearance and blood pressure had renal tubular acidosis; two (one of whom also had incomplete renal tubular acidosis had reduced urine concentrating ability (Elisaf et al. 1993). In a Swedish study, GFR was studied in 66 patients six months after NE, and only 3 patients had a GFR less than 80 ml/min as estimated by ^{51}CrEDTA clearance; two of these, however, had underlying chronic diseases and one had suffered from clinically severe NE (Settergren et al. 1990).

Occasional reports have been published of persistent hypertension in patients following apparent recovery from HFRS (Rubini et al. 1960, Kleinknecht and Rollin 1992). Moreover, serological studies of patients with renal disease in Baltimore have suggested an association between hypertensive renal disease and seropositivity for SEOV (Glass et al. 1990, Glass et al. 1993). Dialysis patients were more likely to have hantavirus antibodies than an age-stratified reference group (2.76 vs. 0.25%, respectively; odds ratio 5.03; confidence interval 1.50 to 17.68): a similar trend was seen in patients with proteinuria (1.46 vs. 0.25% in the reference group) (Glass et al. 1993). In addition, dialysis patients with hantavirus antibodies had hypertension as the principal cause for their renal disease.

A significant increase in the prevalence of antibodies against HNTV and PUUV has also been described in patients with end-stage renal disease from Italy, Greece, Germany and Israel (Nuti et al. 1992, Tsianos et al. 1993, Zöller et al. 1995, George et al. 1998). However, the numbers of patients in these studies were small, and possible cross-reaction between antibodies against hantaviruses and other plasma constituents such as hepatitis B or C antigens cannot be

excluded as an explanation for the high antibody prevalences in the dialysis populations studied (Tsianos et al. 1993).

In contrast to above-mentioned findings, two large seroepidemiological studies from Northern Sweden found no association between previous PUUV infection and cardiovascular disease, renal dysfunction or diabetes mellitus (Ahlm et al. 1994a, Niklasson et al. 1994). Likewise no increases in hantavirus antibody seroprevalence figures have been found among dialysis patients in Belgium, Ireland and the Netherlands (van der Groen et al. 1983, Conlon et al. 1993, Groen et al. 1995b).

A case report has been published of a patient with acute HFRS and diffuse proliferative glomerulonephritis with complete recovery (Grcevska et al. 1990). The association between hantaviral infection and glomerulonephritis in this chance case might be incidental. Acute tubulointerstitial nephritis is a typical histopathological finding in HFRS, and glomeruli are only slightly affected (Collan et al. 1991, Cosgriff 1991, Mustonen et al. 1994b).

Host genetic factors

Since the 1930s, a genetic basis for interindividual variation in human infectious diseases has been indicated by several twin, adoptee, pedigree and candidate gene studies (Hill 2001). Genetic linkage analyses of infectious diseases have yielded convincing evidence of genetic linkage to chromosomal regions where susceptibility genes have yet to be identified (Hill 2001). These studies indicate a distinctly polygenic basis for susceptibility to many common infectious diseases.

Human leukocyte antigens

Human leukocyte antigens (HLA) are encoded on major histocompatibility complex. They are major cell surface antigens, which are specialized for the presentation of antigen peptides to T cells. Class I HLA molecules are expressed on the surface of all cells. They are recognized by CD8+ T cells. Class II HLA

molecules, recognized by CD4+ T cells, are expressed only on the surface of specialized antigen presenting cells.

Viral infectious diseases

Evidence of association of HLA with the rate of progress of human immunodeficiency virus (HIV)-infection has been provided by several studies. HLA-B*5701 was found in 11 out of 13 non-progressors with low viral loads, but in only 10% of controls (Migueles et al. 2000). In a U.S. study, HLA-B*35 and Cw*04 were connected to rapid progression to acquired immune deficiency syndrome (AIDS)-defining illnesses (Carrington et al. 1999). Furthermore, the extended HLA haplotype containing alleles A1, B8, and DRB1*0301 has been associated with rapid progression, and HLA B27 antigen with slow progression of HIV disease (McNeil et al. 1996).

Several European studies have shown an association between the linked HLA class II alleles, HLA-DRB1*11 and HLA-DQB1*0301, and resistance to persistent HCV infection (Alric et al. 1997, Thursz et al. 1999, Zavaglia et al. 1998). On the other hand, HLA-DRB1*0301 and DQB1*0201 alleles have been associated with persistent HCV infection in a Thai population (Vejbaesya et al. 2000).

Nephropathia epidemica

Host genetic factors have been shown to influence the highly variable clinical course of NE. Serological HLA A, B and Cw, PCR-typed DRB1, DQA1, DQB1, DPB1 alleles, as well as complement component C4 allotypes, were determined in 74 adult hospital-treated patients with NE (Mustonen et al. 1996). The investigators found that patients suffering from the most severe outcome of NE frequently carried the haplotype containing HLA B8, C4A*Q0 (C4 null allele), DRB1*0301, DQA1*0501 and DQB1*0201 alleles. There is a very high linkage disequilibrium between the alleles of this haplotype (Alper et al. 1992), and the haplotype is usually inherited as a fixed combination. Seven out of 74 patients were in shock on admission, and all carried this haplotype (Mustonen et al.

1996). The haplotype also correlated strongly with the severity of acute renal failure. Part of this material was included in Studies I and II of this thesis.

It has been found, moreover, that the HLA B27 allele associates with a benign clinical course of NE (Mustonen et al. 1998a). This was especially shown in a short duration of hospital treatment time. It is noteworthy that only six out of the 74 (8%) studied hospital-treated patients with NE had this haplotype, that is only half the number expected in the Finnish reference population (17%).

Cytokine gene polymorphism

Changes (polymorphisms) in the cytokine genes may determine the amount of cytokine produced in the inflammatory reaction. This raises the possibility that an innate cytokine profile after inflammatory insults substantially influences the outcome in infectious diseases.

Interleukin-1 family genes

The genes for IL-1α and IL-1β are located on the long arm of chromosome 2, in close linkage with the IL-1Ra gene (Figure 2). All three genes are polymorphic (Cox et al. 1998). In the IL-1β gene there are at least two biallelic base-exchange polymorphisms: at position -511 in the promoter region (di Giovine et al. 1992) and at position +3953 at the 5th exon site (Pociot et al. 1992). Both of these polymorphisms are caused by C (cytosine) to T (thymidine) (allele 2) transitions (di Giovine et al. 1992, Pociot et al. 1992). IL-1β (+3953) allele 2 has been associated with high lipopolysaccharide-stimulated IL-1β responses in *in vitro*-cultured monocytes (Pociot et al. 1992).

In the IL-1α gene there is a base exchange polymorphism at position –889 in the promoter region caused by the C to T (allele 2) transition (McDowell et al. 1995). The frequency of the more rare allele 2 has been shown to be increased in juvenile rheumatoid arthritis (McDowell et al. 1995). In addition, two

microsatellite polymorphisms termed “222/223” and “gz5/gz6” have been located in the IL-1α gene (Cox et al. 1998).

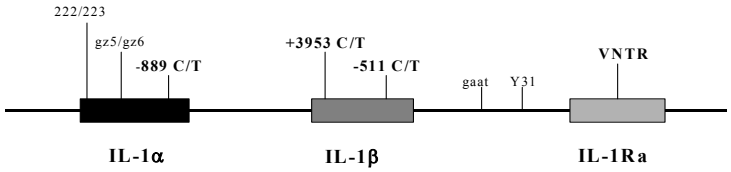

Figure 2. A schematic presentation of the IL-1 gene family in chromosome 2 with known polymorphic loci. The polymorphisms studied in this thesis are shown in bold letters. Based on Cox et al. (1998).

The polymorphism at the IL-1Ra gene is caused by variable numbers of an (86-bp) tandem repeat sequence located in the second intron of the gene. The most common allele 1 contains 4 repeats and allele 2 contains 2 repeats (Tarlow et al. 1993). The IL-1Ra allele 2 has been linked to high secreted IL-1Ra production in *in vitro*-stimulated monocytes (Danis et al. 1995).

The frequency of IL-1Ra allele 2 has been found to be increased in several autoimmune diseases (Hurme et al. 1998). In systemic lupus erythematosus (SLE), carriage of this allele appears to influence the severity of disease rather than susceptibility (Blakemore et al. 1994). The frequency of IL-1Ra allele 2 has also been shown to be significantly higher in patients with severe sepsis than in a normal population (Fang et al. 1999). Two studies have recently reported preliminary evidence that a polymorphism in the IL-1β and the flanking IL-1Ra genes may affect either risk or clinical presentation of tuberculosis (Bellamy et al. 1998, Wilkinson et al. 1999). Furthermore, interleukin-1 gene cluster polymorphisms suspected of enhancing production of IL-1β are associated with

an increased risk of both hypochlorhydria induced by *Helicobacter pylori* and gastric cancer (El-Omar et al. 2000).

Since IL-1Ra is an anti-inflammatory cytokine, there is an apparent paradox in the association of the allele with increased production of IL-1Ra and inflammatory diseases. However, IL-1Ra allele 2 is strongly associated with an unknown allele causing increased IL-1 production *in vitro* (Santtila et al. 1998). Healthy IL-1Ra allele 2 carriers have higher IL-1Ra plasma levels than non-carriers, but the enhancing effect of IL-1Ra allele 2 on IL-1Ra plasma levels requires the presence of the more rare IL-1β (-511) allele 2 or the absence of the more rare IL-1β (+3953) allele 2 (Hurme and Santtila 1998). Cox and co-workers (1998) have identified a common eight-locus haplotype of the IL-1 gene cluster. Their data indicate that IL-1α (-889) allele 1, IL-1β (+3953) allele 1, IL-1β (-511) allele 2 and IL-1Ra allele 2 belong to the same haplotype. It would thus seem plausible that the alleles determining high agonist production and high antagonist production are generally associated.

Tumor necrosis factor alpha gene

The TNFα gene is located within the Class III region of the major histocompatibility complex on human chromosome 6p21, 250 kb centromeric to HLA-B and 850 kb telomeric to HLA-DR (Carroll et al. 1987) (Figure 3). Several polymorphisms have been identified within the promoter region of the TNFα gene (Hajeer and Hutchinson 2001). Among these, there is a single-nucleotide polymorphism at position -308, involving the substitution of guanine (G) by adenosine (A) in the uncommon allele TNF2 (Wilson et al. 1992). This latter TNF2 allele has been linked to enhanced TNFα transcriptional activity (Kroeger et al. 1997, Wilson et al. 1997), but conflicting results have also been published (Stuber et al. 1995, Brinkman et al. 1996).

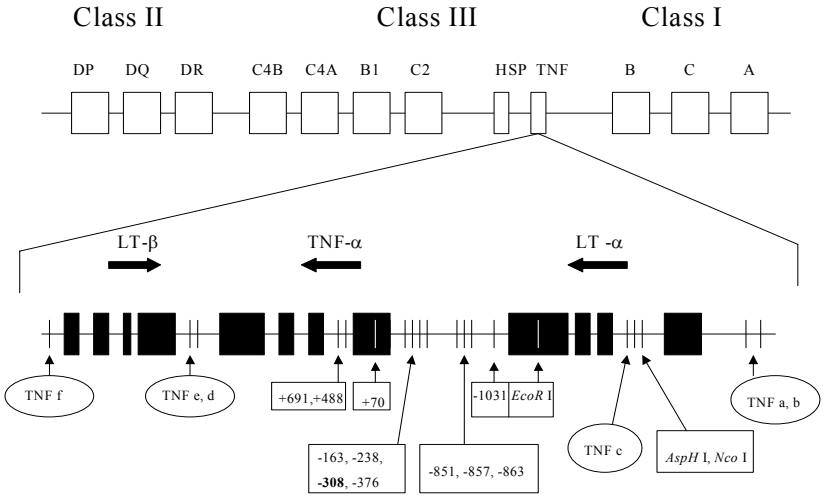

Figure 3. TNF locus relative to the major histocompatibility complex on chromosome 6. Two types of polymorphisms have been found in the TNF locus: microsatellite repeats (circled) and single nucleotide polmorphisms (boxed). The polymorphism studied in this thesis is shown in bold letters. Based on Hajeer et al. (2000). Not in scale.

In Caucasians, TNFα (-308) allele 2 has been associated with susceptibility to SLE, celiac disease and other conditions linked to the extended HLA-B8-DR3 haplotype, and most studies have concluded that the association may be explained by linkage disequilibrium (Wilson et al. 1994, McManus et al. 1996), which is strong especially in Northern European populations (Wilson et al. 1997).

The TNFα (-308) polymorphism has recently been studied in 59 hospital-treated patients with NE (Kanerva et al. 1998b). TNF allele 2-positive patients suffered from a clinically more severe NE than TNF allele 2-negative patients (Kanerva et al. 1998b). These 59 patients are included in the material of this thesis.

AIMS OF THE STUDY

The aims of this study were to establish:

1. whether host genetic factors are associated with susceptibility to nephropathia epidemica
2. whether host genetic factors have an influence on the clinical picture of nephropathia epidemica
3. whether possible changes in cytokine levels in urine and plasma during the acute phase of infection are associated with renal involvement in nephropathia epidemica
4. the long-term prognosis of nephropathia epidemica

SUBJECTS AND METHODS

Subjects

Patients

All patients who participated in this study had suffered from serologically confirmed acute PUUV infection (Hedman et al. 1991, Vapalahti et al. 1995a, Vapalahti et al. 1996a). Studies I-IV were carried out in Tampere University Hospital and in the Medical School, University of Tampere, and all patients and controls were from the Pirkanmaa region. In Study V, one patient was from the Päijät-Häme district and another from Central Finland, while the other three patients lived in the Pirkanmaa area. All patients except for two in Study V were treated in hospital due to acute NE.

Studies I and II included patients hospitalized due to acute NE in three different periods. The first group (Group A) comprised 41 patients treated during the years 1982 to 1993, the second 23 patients treated during the years 1994 to 1995 (Group B), and the third group of 70 consecutive, prospectively included patients treated in the period from September 1997 to December 1999 (Group C). When preparing the manuscript of Study I, cytokine gene polymorphism analyses of 23 patients of Group C were available. Hence only 23 out of the 70 patients of Group C were included in Study I. Studies II and III included 52 and all 70 patients of Group C, respectively. Study IV involved 46 subjects who had suffered from NE 3-7 (mean 5) years previously. They had been hospitalized during the period from October 1990 to September 1995. Only subjects with no

known history of diabetes mellitus, hypertension and renal disease prior to NE were selected. In Study V, five cases with atypical clinical course of NE were described. The number of patients included in Studies I-V is shown in Table 6. The periods when patients of each group were hospital-treated for acute NE are also shown. Clinical characteristics of patients as well as of controls participating in Studies I-V are shown in Table 7.

Table 6. Number of patients participating in Studies I-V. The periods when patients of each group were hospital-treated for acute NE are also shown.

	Group A 41 random-selected patients	Group B 23 consecutive patients	Group C 70 consecutive patients	Group D	Total number
Hospital-treatment during years	1982-93	1994-95	1997-99	1990-95	
Study I	41[a]	23[b]	23[c]		87
Study II	41[a]	23[b]	52[c]		116
Study III			70		70
Study IV	8	12		26	46
Study V					5

a = exactly the same 41 patients, b = exactly the same 23 patients, c = Study II included 18 patients (of Group C) from Study I.

Table 7. Clinical characteristics of patients and controls participating in Studies I-V.

	Study I	Study II	Study III	Study IV	Study V
Number of patients	87	116	70	46	5
Age of patients (years) [a]	43 (15-77)	43 (15-77)	39 (15-70)	44 (23-63)	F66, M58,
Gender of patients M/F	61/26 (70% males)	78/38 (67% males)	49/21 (70% males)	26/22 (57% males)	F43, M29, F44
Controls	400 blood donors	400 blood donors and registry data of 1199 bone marrow donors	400 blood donors (plasma cytokine concentrations)	38 PUUV-seronegative subjects	
Age of controls (years)	18-60	blood donors, 18-60; bone marrow donors, 18-45	18-60	Median 43, (range 27-64)	
Gender of controls M/F	na	na	na	22/16 (58% males)	

na = not available; [a]median (range)

Controls

The control group for cytokine gene polymorphism studies (Studies I and II) and for plasma cytokine concentrations (Study III) comprised 400 healthy blood donors. Plasma IL-1β and IL-6 were measured in all 400 donors, while plasma TNFα and IL-1Ra were analyzed in 200. Blood samples were obtained from the Finnish Red Cross Blood Transfusion Center, Tampere, Finland. The donors were adults (18-60 years of age) and had had no signs of infection during a 2-week period before the blood donation. Healthy bone marrow donors (18-45 years of age) registered with the Finnish Bone Marrow Donor Registry served as controls for HLA studies (Siren et al. 1996), and calculated antigen frequencies of 1199 donors from the Pirkanmaa region were used (Study II). The control group in Study IV comprised 38 healthy PUUV-seronegative subjects. The controls had the same exclusion criteria as the patients: a history of diabetes mellitus, hypertension and renal diseases.

Methods

Study protocols

Consecutive patients admitted to hospital and evincing a clinical picture typical of NE were prospectively included in Study III. A detailed medical history was obtained and a physical examination made during their hospital stay. Urine collection commenced on the first evening of hospital care and continued until three days were completed. The nightly collection period was measured from the time of the last voiding (into the toilet) at bedtime until the last voiding (into a polyethylene plastic receptacle) upon rising. The daytime collection commenced immediately thereafter, and was maintained until the total 24 hours were completed in the evening. The samples were kept in a refrigerator during collection and after completion volumes were measured and timing recorded for the two collection periods. Urine samples were stored at -70°C. The first blood

sample was obtained on the first morning (day 1) of hospital care, whereafter blood samples were taken on two consecutive mornings (days 2 and 3). The samples were collected in sterile EDTA tubes. Plasma was prepared by centrifugation and stored at -70°C. After one year, the patients were invited to follow-up examinations at the outpatient department, and 56 (80%) of them attended. A fractionated 24-h urine collection was carried out as in the acute phase.

Clinical tests in Study IV were carried out from September 1996 to April 1999 at the outpatient department in Tampere University Hospital. A detailed past and current medical history was obtained and a careful physical examination made. Again, a fractionated 24-h urine collection was carried out as described above. On the morning following completion of urine collection, the study subjects visited the laboratory, where a spot sample of second morning urine and blood samples were obtained. The morning urine specimens and blood samples were obtained after a minimum of 12-h fasting. Renal function tests were performed on different days in order to avoid the dehydration which could have resulted from fasting.

Analytical methods

In Studies III and IV, quantitative 24-h urinary protein excretion was measured by the pyrogallol red molybdate method (Olli C, Kone Instruments, Helsinki, Finland). Timed overnight urinary excretion of α1-microglobulin, albumin and IgG was measured by nephelometry (Behring Nephelometer II Analyzer, Behringwerke AG, Marburg, Germany). Overnight urinary excretions of α1-microglobulin ≥7 μg/min, albumin ≥11 μg/min and IgG ≥5 μg/min were considered abnormal based on the healthy reference material of our laboratory.

In Study IV, spot samples of second morning urine were tested with dipsticks. A microscopic urine examination was made if dipstick tests showed positive results for erythrocytes, leukocytes, protein or nitrite. Hematuria was defined as a positive dipstick test for erythrocytes and over two erythrocytes per

high-power field. In Study III, hematuria was defined likewise, but urine samples did not need to be timed.

In Study IV, serum creatinine, urea, sodium, potassium, total cholesterol, high-and low-density lipoprotein cholesterol, triglycerides and blood glucose were determined by Vitros, Johnson & Johnson, Rochester, NY, USA, and blood cell count by Technicon H3, Bayer Diagnostics, Elkhart, IN, USA. In Study III, serum creatinine and other analytical procedures were carried out with an automated chemistry analyzer using routine procedure. In Study V, serum complement component concentrations (C3, C4) were measured by laser nephelometry.

Puumala virus serology

PUUV antibodies were determined by the available methodology used in the Department of Virology, University of Helsinki. As well as in patients, PUUV antibodies were analyzed in the 400 blood donors (Study I) and in the controls of Study IV. During the earliest years, the diagnosis was based only on duplicate samples with a 4-fold (or more) rise in IgG titer detected by IFA. Since July 1989 (Brummer-Korvenkontio et al. 1999), a recent PUUV infection has been confirmed from a single serum sample by detecting the typical granular staining pattern in IFA (Vapalahti et al. 1995a) and/or low avidity of IgG antibodies to PUUV (Hedman et al. 1991) and/or by detecting PUUV IgM antibodies by an "in-house" ELISA based on recombinant antigens (Vapalahti et al. 1996a).

Cytokine gene polymorphism

Genomic DNA for the studies was isolated from mononuclear cells using the salting-out method (Miller et al. 1988). With these, PCR-based genotyping of IL-1β (base exchange polymorphisms at positions -511 and +3953), TNFα (base exchange polymorphism at position -308), IL-1Ra (variable number of tandem repeats in exon 2) and IL-1α (base exchange polymorphism at position -889)

was performed as previously described (di Giovine et al. 1992, Pociot et al. 1992, Wilson et al. 1992, Tarlow et al. 1993, McDowell et al. 1995).

Human leukocyte antigens

In Study II, the HLA-B alleles were determined by the standard microlymphocytotoxity test, and the HLA-DRB1 alleles by group-specific DNA amplification followed by restriction enzyme digestions (Westman et al. 1993). In Study V, the HLA-A and HLA-B antigens were correspondingly determined by the standard microlymphocytotoxity method, except in one patient (patient No. 5), whose HLA-B alleles were determined from genomic DNA using the B locus SSP Unitray kit (Pel-Freez Clinical Systems, Deerbrook Trail, Wisc., USA), since no fresh blood was available for typing by standard method. The HLA-DRB1 alleles of patients in study V were determined using the Inno-LiPa HLA-DR kit (Innogenetics, Zwijndrecht, Belgium).

Cytokines

Plasma and urinary IL-1β, IL-6, IL-1Ra and TNFα concentrations were determined using commercially available ELISAs (PeliKine Compact™ human IL-1β, IL-6 and human TNFα kits, Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service, Amsterdam, The Netherlands, and Quantikine human IL-1Ra immunoassay, R&D Systems, MN, USA) following the manufacturer's instructions. The detection limits for the assays were 0.4 pg/ml for IL-1β and IL-6, 46.9 pg/ml for IL-1Ra, and 1.4 pg/ml for TNFα.

Ambulatory blood pressure

Twenty-four-hour ambulatory blood pressure (ABP) was measured during one 24-h period by the oscillometric and/or auscultatory method using a non-invasive, fully automatic recorder (Novacor Diasys Integra, France). An

experienced nurse fitted the device, and blood pressure was verified against a mercury sphygmomanometer upon installation and removal. At the start of each ABP registration, three measurements were taken with the Novacor Diasys Integra monitor in the outpatient clinic. Thereafter, readings were recorded at 15-min intervals during daytime and at 30-min intervals during the night.

Glomerular filtration rate and effective renal plasma flow

The GFR was determined by the plasma clearance of ^{51}CrEDTA assessed by a single-injection method (Garnett et al. 1967). The ERPF was estimated by clearance of ^{131}I-hippurate.

Renal histology

The renal biopsy specimens were processed for light-microscopic, electron-microscopic and immunofluorescence studies using standard techniques (Mustonen et al. 1994b).

Mathematical formulas

In Study III, timed overnight urinary excretion of cytokines was calculated as follows: (concentration x total volume)/(time span). The highest plasma or urinary cytokine concentration recorded during the first three days was designated the maximum value of the cytokine concentration. Overnight urinary excretion of α1-microglobulin, albumin and IgG below the detection limit for nephelometry was taken as 1 μg/min in calculations. The fractional clearance (Θ) of a protein was calculated using the equation $\Theta_{protein} = (U_{protein} \times P_{creatinine})/(P_{protein} \times U_{creatinine})$, where U is the urinary concentration (of the overnight collection period) and P the plasma concentration.

In Study IV, body mass index (BMI) was calculated by dividing weight (kg) by the square of height (m^2), and FF was calculated as the quotient of GFR and

ERPF. Three blood pressure measurements were taken at the beginning of each ABP registration, and the means of these readings were taken as office blood pressures. Mean values for systolic (SBP), diastolic (DBP) and mean blood pressure (MBP), as well as heart rate were calculated from each registration during the 24-h, day- and night-period. MBP was calculated as follows: DBP + 1/3(SBP-DBP).

Statistical methods

To describe the data, means and standard deviations (SD) were given for normally distributed variables and medians and ranges for skew-distributed continuous variables. For categorical variables percentages were used. Pairwise comparisons were made with independent sample t-tests for normally distributed variables and Mann-Whitney U-tests for skewed variables. Kruskal-Wallis test was used to compare the groups if the groups numbered more than two. Thereafter pairwise comparisons were performed by Mann-Whitney U-test with exact p, where the p values were corrected by the Bonferroni method. Fisher's exact test or χ^2 test was used for categorical data.

In Study I, allelic frequencies (number of copies of a specific allele divided by the total number of alleles in the group) and carriage rates for infrequent 2-alleles (number of individuals with at least one copy of allele 2 divided by the total number of individuals within the group) were calculated in NE patients, and seronegative and -positive controls. χ^2 test was used to assess whether there were differences in the carriage rates between NE patients and seronegative controls, or between the groups in general. Odds ratios (OR) were calculated after the χ^2 test when appropriate, and 95% confidence intervals (95%CI) were determined.

The patients in Studies I and II were grouped into carriers (including both homozygotes and heterozygotes) and non-carriers of specific alleles. The significance of differences in allele frequencies was estimated by χ^2 test. Logistic regression analysis was carried out in Study II to identify which haplotype contributed to variables reflecting the clinical severity of NE.

In Study III, Wilcoxon's test was used to evaluate changes in urinary cytokine excretion between the acute phase of NE and after one year. Changes in time (days 1-3) were analyzed using analysis of variance for repeated measurements. Analyses were performed after logarithmic transformation of the data. The highest urinary cytokine excretion measured during days 1-3 was correlated with that urinary protein excretion or plasma cytokine concentration which was measured on the same day when the maximum urinary cytokine excretion was measured. Correlations were calculated by Spearman rank correlation coefficient.

In Study IV, Pearson's correlation coefficient was used to examine relationships between normally distributed continuous variables. Skewly distributed variables were logarithmically transformed before analysis. Multiple linear regression analysis was carried out to identify factors determining proteinuria in the patient group. Analysis of covariance was made to determine whether the difference in proteinuria and GFR between patients and controls could be ascribed to a difference in factors other than the previous NE disease.

All testing was two-sided and the limit of significance was set equal to 0.05. Computation was carried out using SPSS for Windows statistical software (version 7.0).

Ethical considerations

All subjects gave written informed consent before participation and the study protocols were approved by the Ethics Committee of Tampere University Hospital.

RESULTS

The clinical picture (Studies I-IV)

The clinical picture was typical of acute NE in all patients participating in Studies I-IV. Table 8 shows laboratory and clinical findings in patients of 3 groups (Groups A, B and C) formed according to time period when treated at Tampere University Hospital. Patients of Group C had a lower peak median serum creatinine concentration measured during hospital care and shorter treatment time in hospital than the patients of Groups A and B. Those in Groups A and B required transient dialysis treatment more often than the patients in Group C. The clinical picture of NE did not differ between genders in the whole patient group or within the Groups (data not shown). All patients recovered.

Table 8. Clinical characteristics of patients with NE treated in different periods in Tampere University Hospital.

	Group A, n=41	Group B, n=23	Group C, n=70	p
Hospital-treatment (during years)	1982-93	1994-95	1997-99	
Age (years)[a]	43 (20-76)	44 (26-77)	39 (15-70)	0.297
Gender (M/F)[b]	29/12	15/8	49/21	0.889
Patients in shock[b]	4 (10%)	3 (13%)	3 (4%)	0.305
Patients requiring dialysis[b]	5 (12%)	8 (35%)	3 (4%)	<0.001
Treatment time (days)[a]	8 (5-46)	8.5 (3-28)	7 (3-13)	<0.001
S-$Crea_{max}$ (μmol/l)[a]	298 (93-1537)	319 (93-1156)	232 (76-878)	0.023
B-$Leuk_{max}$ ($x10^9$/l)[a]	10.8 (4.5-50.3)	11.0 (6.0-37.7)	10.2 (4.4-23.4)	0.389
B-$Thromb_{min}$ ($x10^9$/l)[a]	70 (9-362)	64 (11-178)	63 (3-198)	0.244
Change in weight (kg)[a]	2.9 (0.5-12.9)	3.2 (0.1-16.0)	2.6 (0-9.9)	0.348
SBP_{min} (mmHg)[a]	120 (50-155)	120 (60-150)	112 (85-157)	0.399

[a]median (range), Kruskal-Wallis test, [b]numbers (%), χ^2 test, S-$Crea_{max}$= the highest serum creatinine concentration, B-$Leuk_{max}$= the highest blood leukocyte count, B-$Thromb_{min}$ = the lowest blood thrombocyte count, SBP_{min} = the lowest systolic blood pressure measured during hospital care.

In the patients in Study III (Group C) the median duration of fever was 5 days (range, 2 to 14 days), and the median interval between the onset of symptoms (i.e. fever) and admission was 4 days (range 2 to 15 days). The diagnosis of referral was correct in 45 out of 70 (64%) cases. Three (4%) patients had hypotension (SBP≤90 mmHg) on admission, and one of them also needed dialysis treatment. Altogether 3 (4%) patients were dialysed. Four (6%) patients had oliguria (urine output <400 ml/day), while polyuria (>3000 ml/day) was noted in 79% of patients in the convalescent phase. Proteinuria (>0.3 g/d) was detected in 65 out of 70 (93%) patients being of nephrotic (>3.5 g/d) range in 33% of them. Hematuria was deteced in 94% of patients. Acute renal failure (maximal serum creatinine detected during hospital care >100 μmol/l) occurred in 55 (79%) patients, and was regarded as severe (maximal serum creatinine ≥500 μmol/l) in 9. Thrombocytopenia (the lowest blood thrombocyte count measured ≤150 x 10^9/l) and hypoalbuminemia (the lowest plasma albumin concentration ≤ 36 g/l) were both seen in 69 out of 70 patients. Seventeen (24%) patients received empirical antibiotics during the hospital stay. Venous blood for bacteriological culture was collected from 59 (84%) patients. All were negative.

In Study IV, acute impairment of renal function had been observed in 85% of the patients during acute NE. The highest serum creatinine concentrations found during hospital care had ranged from 75 to 1645 μmol/l (median 237), and 5 out of 46 (11%) patients had required transient dialysis therapy.

Host genetic factors (Studies I and II)

Polymorphisms of the interleukin-1 family genes (Study I)

Study I brought out no statistically significant differences in distributions of the IL-1α, IL-1β (-511), IL-1β (+3953) and IL-1Ra alleles in patients compared to controls. Among the 400 healthy blood donors there were 19 (5%) PUUV-seropositive persons. The carriage rates for infrequent 2-alleles of IL-1β (-511)

and IL-1Ra in NE patients, PUUV-seronegative and -seropositive blood donors are shown in Table 9. A tendency towards a decreased frequency of IL-1β (-511) allele 2 was observed in patients compared to seronegative controls. In comparison of the carriage rates of IL-1β (-511) allele 2 between all three groups (hospitalized NE patients, PUUV-seronegative and -seropositive blood donors), a statistically significant difference was found.

Table 9. Carriage rates for infrequent 2-alleles of IL-1Ra and IL-1β (-511) in hospitalized NE patients, PUUV-seronegative and -seropositive blood donors, χ^2 test.

	NE patients (n=83)	Seronegative blood donors (n=381)	Seropositive blood donors (n=19)	p
IL-1β 2-carriers	53%	62%	84%	0.033
IL-1Ra 2-carriers	42%	48%	63%	0.244

The IL-1Ra allele 2 was strongly associated with IL-1β (-511) allele 2 both in NE patients and in PUUV-seronegative controls ($p<0.001$ in both groups). As shown in Table 10, however, the allele associations differed between the groups, the frequency of non-carriers of IL-1Ra allele 2/IL-1β (-511) allele 2 being increased in NE patients compared to PUUV-seronegative blood donors (38% vs. 27%). Only two out of 19 (11%) PUUV-seropositives were IL-1Ra 2-negative/IL-1β 2-negative.

Table 10. Associations between the alleles of the IL-1β (-511) and IL-1Ra in NE patients and in PUUV-seronegative blood donors.

		IL-1β (-511) 2-positive	IL-1β (-511) 2-negative
NE patients (n=81)	IL-1Ra 2-positive	28 (35%)	6 (7%)
	IL-1Ra 2-negative	16 (20%)	31 (38%)[a]
Seroneg. donors (n=381)	IL-1Ra 2-positive	144 (38%)	38 (10%)
	IL-1Ra 2-negative	95 (25%)	104 (27%)[a]

a= hospitalized NE patients and PUU virus-seronegative blood donors, OR 1.65, 95%CI 1.0-2.7.

Tumor necrosis factor alpha gene polymorphism and human leukocyte antigens (Studies I and II)

In Study I there was a tendency towards an increased frequency of "high-producer" TNFα allele 2 between patients and seronegative controls, but the difference was not statistically significant. Study II included 82 patients from Study I with an additional 34 patients. In this larger material the frequency of TNFα allele 2 was 42.2% in patients and 31.3% in blood donors. The allele frequencies of HLA-B8 and DRB1*0301 (DR3) in 116 hospitalized NE patients were increased compared to controls (32.8% vs. 16.8% and 31.9% vs. 17.6%, respectively). The frequency of HLA-B27 was decreased among patients compared to controls (6.9% vs. 14.4%).

The alleles HLA-B8 and -DR3 were strongly associated with each other in the patient group (χ^2 test, $p<0.001$), and these alleles were therefore combined (B8-DR3) in further analyses. Altogether 49 patients were TNF2-carriers. The TNF2 allele was associated with the HLA-B8-DR3 haplotype (χ^2 test, $p<0.001$) (Table 11).

Table 11. The distribution of B8-DR3 and TNF2 alleles among 116 patients with NE.

	B8-DR3-positive	B8-DR3-negative	total
TNF2-positive	31	18	49
TNF2-negative	4	63	67
total	35	81	116

Influence on clinical course (Studies I and II)

In Study I involving 87 hospitalized patients, several clinical and laboratory parameters showed a more severe clinical course of NE in TNF2-carriers (genotypes 1.2 and 2.2) than in non-carriers (genotype 1.1). The clinical severity of NE did not differ either between IL-1β 2-carriers and non-carriers, between IL-1Ra 2-carriers and non-carriers or between the IL-1Ra 2-negative/IL-1β 2-negative and the other patients.

As expected, TNF2-carriers evinced a tendency towards a more severe disease compared to non-carriers also in the 116 patients in Study II. However,

the differences in clinical findings between HLA-B8-DR3-positives and -negatives were statistically more significant than those between TNF2-carriers and non-carriers. Ten out of 116 (9%) patients were in clinical shock (SBP ≤90 mmHg with clinical signs and symptoms of shock) on admission. All had the HLA-B8 and -DR3 alleles and 9 of them were also TNF2-carriers. Almost all (33 out of 35) B8-DR3-positive patients had acute impairment of renal function (maximum serum creatinine exceeding 150 μmol/l measured during the acute phase of NE) compared to 58% of B8-DR3-negative patients (p=0.001). Correspondingly, 36 out of 49 (74%) TNF2-carriers suffered acute impairment of renal function, compared to 44 out of 67 (66%) TNF2-non-carriers (p=0.370).

Four patient groups were formed in respect of carriage or non-carriage of TNF2 and B8-DR3. Maximum serum creatinine concentration and blood leukocyte count were higher in TNF2-positive/B8-DR3-positives than in TNF2-positive/B8-DR3-negatives, or in TNF2-negative/B8-DR3-negatives. The lowest DBP was lower in TNF2-positive/B8-DR3-positives than in TNF2-negative/B8-DR3-negatives. The peak serum CRP concentration was lower in TNF2-positive/B8-DR3-positives than in TNF2-positive/B8-DR3-negatives. There were, however, no differences in the clinical severity of NE in 18 patients who were TNF2-positive/B8-DR3-negative compared to 63 who were TNF2-negative/B8-DR3-negative. All four patients who were TNF2-negative/B8-DR3-positive seemed to have a rather severe disease.

Only 3 patients (in Study II) were homozygous for TNF2 allele. All suffered from severe NE: two of them were dialysed and all developed marked renal insufficiency (maximal serum creatinine ranging from 714 to 987 μmol/l). They also had the HLA-B8 and -DR3 alleles.

Sixty-four out of 116 patients in the present study had previously been analyzed in respect of HLA genes (Mustonen et al. 1996). Thereafter DNA and HLA samples of 52 consecutive hospital-treated NE patients were collected. When this group was analyzed separately, it was found that the median maximum serum creatinine measured during the acute phase of NE was higher in B8-DR3-positive than in -negative patients, and again, all 3 patients with shock on admission had HLA-B8 and -DR3 alleles. In this material of 52 patients, no

differences in clinical outcome of NE were seen between TNF2-carriers and -non-carriers (Table 12)

Table 12. Clinical findings in HLA-B8-DR3-positives and -negatives and in TNF 2-carriers and non-carriers in 52 hospitalized patients (from Group C) with NE.

	B8-DR3-pos. (n=16)	B8-DR3-neg. (n=36)	p	TNF2-carriers (n=21)	TNF2-non-carriers (n=31)	p
Patients in shock (n=3) [b]	3	0	0.025	3	0	0.060
Patients requiring dialysis (n=3) [b]	1	2	0.921	1	2	0.798
Change in body weight (kg) [a]	3.4	2.4	0.184	3.2	2.7	0.648
Treatment time in hospital (days) [a]	8	6.5	0.203	8	7	0.962
S-$Crea_{max}$ (μmol/l) [a]	389	149	0.006	208	213	0.780
B-$Leuk_{max}$ ($x10^9$/l) [a]	12.2	9.7	0.074	11.1	10.2	0.198

[a]median, Mann-Whitney U-test; [b]χ^2 test. S-$Crea_{max}$ = the highest serum creatinine concentration, B-$Leuk_{max}$ = the highest blood leukocyte count measured during hospital care

Table 13 shows results of logistic regression analysis with the severity of renal failure and the amount of leukocytosis as dependent variables, and HLA-B8-DR3 and TNF2 positivity/negativity as independent variables. The significant factor contributing to the clinical severity of NE turned out to be B8-DR3.

Table 13. Logistic regression analysis of HLA-B8-DR3 positivity/negativity and TNFα (-308) allele 2 positivity/negativity in relation to the severity of renal failure and the amount of leukocytosis in 116 hospitalized NE patients.

	p	OR	95%CI
Dependent variable: S-$Crea_{max}$ <500 or ≥500 μmol/l (n1/n2=89/27)			
HLA-B8-DR3	0.008	5.6	1.9-16.3
TNF2 allele	0.559	0.7	0.2-2.0
Dependent variable: B-$Leuk_{max}$ <10.0 or ≥10.0 $x10^9$/l (n1/n2=49/65)			
HLA-B8-DR3	0.027	3.4	1.4-8.6
TNF2 allele	0.654	0.8	0.4-1.8

S-$Crea_{max}$ = the highest serum creatinine concentration, B-$Leuk_{max}$ = the highest blood leukocyte count measured during hospital care

Only 8 out of 116 patients with NE were HLA-B27-positive. The clinical severity of NE did not differ between HLA-B27-positives and -negatives (Table 14). B27-positives were younger than B27-negatives and all of them were men. However, the severity of NE did not correlate with age in the whole patient group and no differences in clinical picture were noted between genders (data not shown).

Table 14. Clinical findings in HLA-B27-positives and -negatives in 116 hospitalized patients with NE.

	B27-positives (n=8)	B27-negatives (n=108)	p
Age (years) [a]	35	44	0.022
Gender (M/F) [b]	8/0	70/38	0.041
Patients in shock (n=10) [b]	0	10	0.368
Patients requiring dialysis (n=16) [b]	0	16	0.241
Treatment time in hospital (days) [a]	7.5	8	0.298
S-$Crea_{max}$ (µmol/l) [a]	275	266	0.983
B-$Leuk_{max}$ (x10^9/l) [a]	9.6	10.6	0.837

[a]median, Mann-Whitney U-test; [b]χ^2 test. S-$Crea_{max}$ = the highest serum creatinine concentration, B-$Leuk_{max}$ = the highest blood leukocyte count measured during hospital care.

Cytokines and other proteins (Study III)

Plasma cytokine levels

As shown in Figure 4, maximum plasma IL-1β, IL-6, IL-1Ra and TNFα concentrations were increased during the acute phase of NE compared to those in healthy blood donors. The plasma concentrations of IL-6 and IL-1Ra declined during the 3 days in hospital.

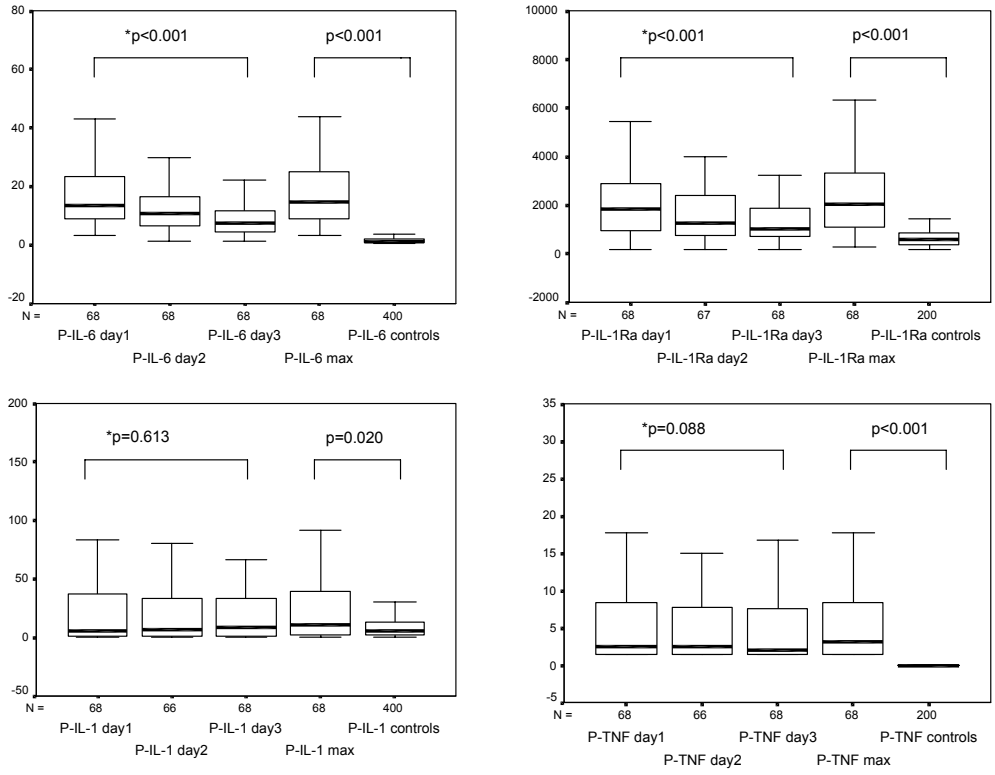

Figure 4. Plasma IL-6, IL-1Ra, IL-1β and TNFα levels (pg/ml) in patients with NE during the first 3 days in hospital. *Change over time. The maximum (max) median cytokine concentration is compared to that in controls. Extremes and outliers are omitted.

Urinary excretion of cytokines and other proteins

The peak overnight urinary excretion of IL-6, IL-1Ra, IL-1β and TNFα (Figure 5), as well as that of albumin, IgG and α1-microglobulin excretion and 24-h protein excretion was increased during the acute phase of NE compared to the corresponding values after one year.

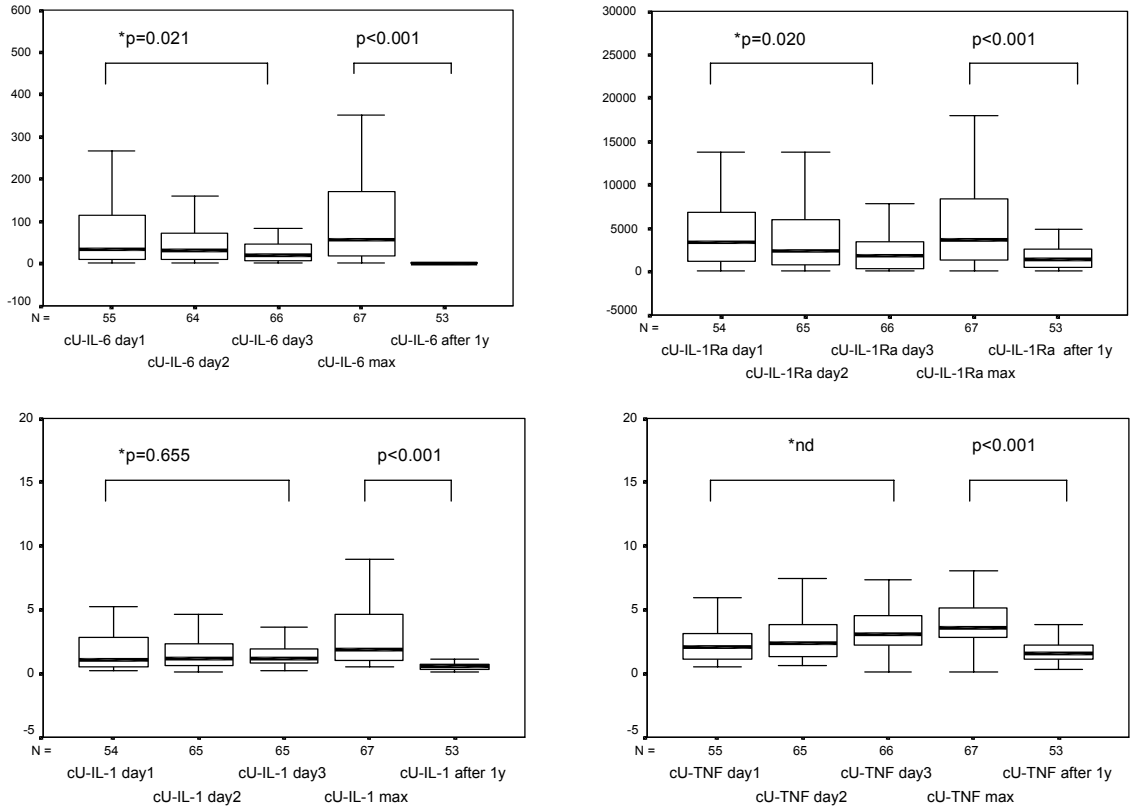

Figure 5. Overnight urinary excretion (pg/min) of IL-6, IL-1Ra, IL-1β and TNFα in patients with NE during the first 3 days in hospital. *Change over time. The maximum (max) median cytokine excretion during the acute phase of NE is compared to that after one year. nd=not done (due to large number of urinary TNFα concentrations below the detection limit of the assay). Extremes and outliers are omitted.

Table 15 shows that the highest urinary excretion of IL-6, IL-1Ra, albumin and IgG was measured on the first day, whereafter the levels declined. The fractional clearances of albumin and IgG declined correspondingly during days 1-3, but the fractional clearances of IL-6 and IL-1Ra were not altered.

Table 15. Excretion of urinary proteins and cytokines, and fractional protein and cytokine clearances in patients with NE during the first three days in hospital. Values are medians (ranges), analysis of variance for repeated measurements.

	N	Day 1	Day 2	Day 3	p
cU-IL-6 (pg/min)	52	36.9 (0.5-562.8)	30.1 (0.3-884.2)	19.1 (0.7-241.5)	0.023
ΘIL-6 ($x10^{-3}$)	48	48.3 (0.8-1546.5)	64.7 (0.6-4167.7)	59.7 (0.9-752.1)	0.595
cU-IL-1Ra (pg/min)	52	3467 (94-60133)	2435 (31-63686)	2118 (66-56829)	0.020
ΘIL-1Ra ($x10^{-3}$)	47	38.5 (0.8-287.2)	29.7 (0.1-1747.6)	20.3 (0.3-2901.6)	0.895
cU-albumin (μg/min)	48	739 (4-7026)	408 (3-5793)	225 (1-3229)	<0.001
ΘAlb ($x10^{-6}$)	43	459 (1-12008)	286 (1-20103)	104 (1-27495)	<0.001
cU-IgG (μg/min)	49	120 (1-1211)	81 (1-1565)	40 (1-1406)	<0.001
ΘIgG ($x10^{-6}$)	44	352 (3-9911)	208 (2-22562)	58 (2-26082)	<0.001

Θ = fractional clearance of protein

Correlations

The maximum urinary IL-6, as well as maximum IL-1β excretion, correlated significantly with urinary albumin ($r=0.79$, $p<0.001$ and $r=0.72$, $p<0.001$, respectively) and protein excretion ($r=0.65$, $p<0.001$ and $r=0.66$, $p<0.001$, respectively). There was also a positive correlation between the maximum urinary IL-6 and IgG excretion ($r=0.76$, $p<0.001$), as well as between IL-1β and IgG excretion ($r=0.69$, $p<0.001$). Peak urinary IL-1β excretion also correlated with the highest plasma levels of IL-1β ($r=0.47$, $p<0.001$), while no correlation between maximum urinary IL-6 excretion and peak plasma IL-6 levels was seen ($r=0.18$, $p=0.148$). Maximum urinary IL-1β excretion also correlated slightly with serum creatinine concentration ($r=0.36$, $p=0.004$).

The maximum urinary IL-1Ra excretion correlated weakly with urinary albumin ($r=0.34$, $p=0.006$), IgG ($r=0.31$, $p=0.015$), protein ($r=0.36$, $p=0.004$) and α1-microglobulin excretion ($r=0.41$, $p=0.001$), and with peak plasma IL-1Ra levels ($r=0.46$, $p<0.001$). The maximum urinary TNFα excretion did not correlate either with urinary albumin, IgG, α1-microglobulin or protein excretion.

Sequelae (Studies IV and V)

Renal function and blood pressure (Study IV)

Study IV revealed no differences in basic laboratory test results between patients who had suffered from hospital-treated NE 3 to 7 years previously, and healthy controls. The groups were also comparable with respect to age, BMI, gender distribution and socio-economic status. All subjects had normal serum creatinine and urea levels. The mean GFR in the patients was increased compared to controls. Thirteen out of 44 (30%) patients had GFR above 130 ml/min/1.73m^2. Also the calculated FF was higher in patients than in controls. ERPF did not differ between the groups.

The patients had significantly higher urinary protein excretion during the entire day than the controls. Nine out of 43 (21%) patients had slightly elevated overnight urinary excretion of α1-microglobulin compared to one of the 38 controls (p=0.012). Urinary albumin excretion was above the normal upper reference limit in 6 (14%) patients and in one control (p=0.114). Further, the patients had higher mean ambulatory SBP than the controls, and also significantly higher MBP than the controls during the 24-hour and day periods and higher heart rate during the whole day.

Table 16 shows 9 patients, who evinced either elevated ABP values (>133/82 mmHg) (Staessen et al. 1994) or a history of hypertension developing after NE. Elevated blood pressure values (>140/90 mmHg) had been measured at a health-care center in two patients (Patients 1 and 3, Table 16) ever since and in one patient (Patient 2) two years after the acute phase of NE, but no medication had been used. A diagnosis of essential hypertension had been made and antihypertensive treatment commenced in one female patient one year after NE (Patient 8). Furthermore, ABP monitoring failed for technical reasons in Patient 9. She had, however, already been noted to be hypertensive at the time of the NE episode, but had used no medication.

Table 16. Results of GFR, 24-h urinary excretion of protein, overnight excretion of α1-microglobulin and albumin, and 24-h ABP recording in 9 patients who showed either elevated ABP values or a history of hypertension developing after NE.

	Gender, age	GFR (ml/min/1.73m^2)	dU-Protein (g/d)	Means 24-h (mmHg)	S-Crea$_{max}$ (μmol/l)	cU-α1-miglo (μg/min)	cU-albumin (μg/min)
1	M 51	107	0.18	137/94	1281	< dl	< dl
2	M 42	138	0.38	135/90	915	< dl	13
3	M 49	140	0.29	142/94	515	< dl	12
4	M 54	119	0.20	136/82	918	< dl	< dl
5	M 44	145	0.22	137/85	1241	10	< dl
6	M 61	103	0.16	152/97	102	14	< dl
7	M 61	121	0.18	139/88	134	< dl	< dl
8	F 45[a]	59	0.18	114/76	93	10	< dl
9	F 53	104	0.20	x	1156	< dl	23

S-Crea$_{max}$ = the highest serum creatinine concentration measured during the acute phase of NE, cU-α1-miglo = urinary α1-microglobulin excretion, dl = detection limit of the assay, [a]used antihypertensive medication during the study, x = ABP monitoring failed due to technical reasons.

Chronic glomerulonephritis (Study V)

In Study V, 5 patients are described who developed peripheral swellings, nephrotic-range proteinuria and microscopic hematuria during the convalescent phase of NE. All had suffered from acute febrile illness compatible with NE 2 to 3 weeks earlier and in all cases a recent PUUV infection was serologically confirmed. Four of them were hypertensive at the time of renal biopsy. Serum creatinine levels ranged from 92 to 524 μmol/l and daily urinary protein excretion from 10.98 to 20.60 g. Serum C3 and C4 levels were decreased in 3 and 2 out of 4 patients, respectively. Antinuclear antibodies, antistreptolysin titer and serology for hepatitis B and C were negative in all. Serum cryoglobulins were negative in all 4 patients tested.

Light microscope examination of the renal biopsy specimens disclosed mesangiocapillary glomerulonephritis (MCGN) in all patients. Increased mesangial matrix and cellularity as well as thickening of the capillary walls together with douple contours of the basement membranes were found. In 4 patients, there were exudative features such as infiltrating polymorphonuclear leukocytes, while the histological finding in renal biopsy in one case was more of type of classical type I MCGN. Glomeruli were found in 4 out of 5

immunofluorescence specimens. All revealed heavy granular and diffuse deposits of complement C1q and C3, mainly in the capillary walls. Immunoglobulin deposits were also found in the glomeruli. In electron microscopy examinations, subendothelial electron-dense deposits and interposed mesangial material containing polymorphonuclear cells and cells of the monocyte-macrophage lineage were seen together with an increase in endocapillary cellularity.

The follow-up period from the renal biopsy ranged from 0.5 to 10 years. The findings at the latest assessment of patients showed that 4 of them were in clinical remission of nephrotic syndrome while one male patient rapidly developed end-stage renal failure resulting in dialysis treatment 3 months after the first symptoms of nephrotic syndrome. At the latest assessment visit, 2 other patients had microscopic hematuria and mild proteinuria (0.18 and 0.30 g/d).

DISCUSSION

Clinical picture

The clinical course was typical of acute NE in all patients participating in Studies I-IV. In Study V, 5 patients were described who developed the nephrotic syndrome during the convalescent phase of an otherwise typical acute NE.

The cohort in Study III comprised 70 prospectively included consecutive patients treated in our hospital during the years 1997-99. The most common laboratory findings were thrombocytopenia and hypoalbuminemia, both seen in 98% of patients. Proteinuria, hematuria and acute renal failure were detected in 93%, 94% and 79% of patients, respectively. The clinical picture of NE patients treated in recent years seemed to be milder than that seen in patients treated in earlier periods, especially as far as acute renal failure is concerned. The peak serum creatinine concentration measured during hospital care was significantly lower in patients in the latest treated group than in former groups. The other variables commonly used to describe the clinical severity of NE did not differ between the groups. One explanation for this trend might be increasing knowledge of NE among doctors in public health, possibly resulting in early and considered referrals. This is supported by the finding that in the present study, 64% of the initial diagnoses in referrals were correct compared to 28% of diagnoses in referrals in the 1980s (Mustonen et al. 1994a). Further, a study under way in a hospital is naturally also recognized in the emergency room, which might possibly mean more admissions than normally. Mild cases would thus also be hospitalized.

Host genetic factors

In Study II the previous finding that clinically severe NE is strongly associated with HLA alleles B8 and DR3 was confirmed (Mustonen et al. 1996). Sixty-four patients in the previous study were included in the present series. Thus the observed differences between B8-DR3-positives and -negatives were predictable. However, the present study supported the previous findings, since the B8 and DR3 alleles also were related to shock and severe renal failure in 52 patients not included in the former study. Furthermore, the HLA-B8 and -DR3 allele frequencies in hospitalized NE patients were increased compared to those reported in the general Finnish population (Siren et al. 1996). Since only the most severe NE cases are hospitalized, the over-representation of B8 and DR3 alleles in this material plausibly reflects the association between severe NE and the HLA-B8-DR3 haplotype.

The mechanisms of the associations between severe NE and the HLA-B8-DR3 haplotype are obscure, but emphasize the role of host immune response in the pathogenesis of NE. The antiviral response may be dysfunctional and fail to clear the virus. Such a theory is supported by the finding that PUUV RNA, analyzed by reverse transcription-PCR, is more easily detected in blood and urine samples from HLA-B8-DR3-positive than in B8-DR3-negative patients during the acute infection (Plyusnin et al. 1997a). The antiviral response may also act inappropriately, mimicking autoimmune reactions and hence may contribute to the pathogenesis. It is of interest that the HLA-B8-DR3 haplotype is associated with certain autoimmune diseases such as SLE (Schur 1995) and celiac disease (Sollid et al. 1989).

In Study II, the HLA-B8-DR3 haplotype was strongly associated with hypotension and severity of renal failure. In addition, leukocytosis, thrombocytopenia, anemia and abnormalities in chest roentgenography were associated with these alleles. Unexpectedly, peak serum CRP concentrations were lower in B8-DR3-positives than in negatives. The explanation could be that CRP is primarily derived via IL-6-dependent hepatic biosynthesis, and IL-6 production by blood leukocytes has been shown to be either similar (Candore et

al. 1994) or decreased (Price et al. 1999) in HLA-B8-DR3-positive compared to negative subjects.

It has previously been shown that TNFα (-308) allele 2 carriers suffer from a more severe NE than do TNF2 non-carriers (Kanerva et al. 1998b). The same result was also predictably found in Study I, which included the 59 patients from the earlier study. In Study II, which had more patients than Study I, the differences in variables reflecting the clinical picture of NE between TNF2 carriers and non-carriers were not as significant as in Study I. In Study II the frequency of TNFα (-308) allele 2 was increased in hospital-treated patients with NE compared to healthy blood donors. These observations were plausibly attributable to the strong linkage disequilibrium with the HLA-B8-DR3 haplotype. Such a theory is supported by several findings. First, the differences in clinical outcome of NE between HLA-B8-DR3-positives and -negatives were much more significant than those between TNF2-positives and -negatives. Secondly, there were no differences in clinical severity when TNF2-positives but B8-DR3-negatives were compared to TNF2- and B8-DR3-negative individuals. Logistic regression analysis also confirmed the importance of HLA-B8-DR3 while the TNF2 allele showed no independent effect on outcome variables.

In Caucasians, the TNF2 allele is associated with susceptibility to SLE, celiac disease and other conditions linked to the extended HLA-B8-DR3 haplotype, and most studies have concluded that the association may be explained by linkage disequilibrium (Wilson et al. 1994, McManus et al. 1996). There are only few studies which have elucidated the controversial issue regarding the interdependence of HLA-DR3 and TNF2 alleles. In a Dutch study, Rood and colleagues (2000) demonstrated that the TNF2 allele association with SLE is independent of HLA-DR3, which would imply that this allele may be an independent risk factor. In Africans, the TNF2 allele is seen in individuals not carrying elements of the extended HLA haplotype, and correlates with susceptibility to SLE in African Americans (Sullivan et al. 1997) and to cerebral malaria in Gambia (McGuire et al. 1994), suggesting that the polymorphism can be effective *in vivo*. The TNF2 allele has also been associated, independently of HLA specificities, with lepromatous leprosy (Roy et al. 1997) and

mucocutaneous leishmaniasis (Cabrera et al. 1995). Recently, Mira and associates (1999) demonstrated that the TNF2 allele occurs with increased frequency in patients with septic shock compared to a group of blood donors. In addition, they showed that the mortality due to septic shock was increased in patients with this allele, and every patient who was TNF2 homozygous had a fatal outcome.

It is possible that genetically determined TNFα production still has a role in the clinical course of PUUV infections. There could be another gene which either alone or with the TNFα (-308) G/A polymorphism might be responsible for the "high producer" phenotype. In Study II, three TNF2 homozygous patients evinced marked renal failure requiring transient dialysis therapy during acute-phase NE. The independent significance of homozygosity for TNF2 allele cannot be further evaluated, since all three TNF2 homozygous patients also had the HLA-B8 and -DR3 alleles.

The frequency of the HLA-B27 allele was significantly lower in hospital-treated patients with NE than in the reference population. This might suggest that individuals with B27 have had a clinically mild NE, which is often treated at home. Previously, the HLA-B27 allele has been shown to be associated with a mild course of NE among hospital-treated patients (Mustonen et al. 1998a). In the present series no statistically significant differences in the clinical severity of NE were found between HLA-B27-positive and -negative patients. It should be noted, however, that the sample of B27-positive patients (only eight individuals) was too small to draw any firm conclusions. Interestingly, there is evidence for a decreased production of TNFα and IFN-γ by T cells in HLA-B27-positive patients with ankylosing spondylitis and also in HLA-B27-positive healthy controls, the mechanism of which is unknown (Rudwaleit et al. 2001).

In Study I, the frequency of non-carriers of IL-1Ra allele 2 and IL-1β (-511) allele 2 was found to be increased in the hospitalized NE patients (38%) compared to the group of PUUV-seronegative blood donors (27%), which would suggest that IL-1Ra allele 2/IL-1β (-511) allele 2 polymorphism may contribute to susceptibility to NE. Only 2 out of 19 (11%) PUUV-seropositive controls were IL-1Ra 2-negative/IL-1β 2-negative. Since about 70% of PUUV infections

in Finland remain without serological diagnosis due to subclinical or mild disease (Brummer-Korvenkontio et al. 1999), it is reasonable to assume that most of these seropositive blood donors had had mild infection. On the other hand, hospital-treated patients with NE had obviously suffered from more severe disease. Hence, the result here would also suggest that IL-1Ra allele 2/IL-1β (-511) allele 2 polymorphism may be associated with the clinical severity of NE. However, there were no differences in the clinical picture of NE between the IL-1Ra 2-negative/IL-1β 2-negative and the other patients.

As described in the review of the literature, IL-1β (-511) allele 2 has been found to be significantly associated with the presence of IL-1Ra allele 2 (Cox et al. 1998, Hurme and Santtila 1998). A strong association between these alleles was also observed in the present study. There is an increasing body of data to show that the combinations of the IL-1β and IL-1Ra alleles are of clinical significance, but the mechanisms of these interactions and their effect e.g. on the quantity of cytokines produced are as yet poorly characterized. In inflammatory bowel diseases the association between the IL-1β and IL-1Ra alleles is different from that in healthy individuals (Bioque et al. 1995, Heresbach et al. 1997). Further, in Epstein-Barr virus infection a significantly higher number of non-carriers of IL-1Ra allele 2/IL-1β (-511) allele 2 have been found among Epstein-Barr virus-seronegative than in seropositive blood donors (Hurme and Helminen 1998).

Cytokines

Peak plasma levels of IL-6, IL-1Ra, IL-1β and TNFα were increased in patients with acute NE compared to those in healthy blood donors. These findings are in accord with previous findings reported by Linderholm and associates (1996). They found elevated plasma levels of TNFα and IL-6 in all studied 15 patients with acute NE. Patients were hospitalized within 4 days after the onset of fever and peak levels of TNFα were seen on days 3-5 after onset. In the present series the highest cytokine levels were probably missed, since the interval between the

onset of symptoms and admission to the hospital ranged from 2 to 15 days. When looking at the delay between the onset of the symptoms and collection of the first cytokine samples (3 to 16 days), obviously one extra day was missed due to diagnostic studies in the emergency room. Plasma IL-6 and IL-1Ra levels decreased during the first 3 days in hospital, which supports the idea that the peak had occurred before admission.

Increased plasma cytokine levels in NE were expected findings, since plasma proinflammatory cytokine levels increase non-specifically in acute inflammatory and infectious diseases (Bienvenu et al. 2000). Among the proinflammatory cytokines, however, IL-6 is thought to be the best systemic reflection of the activation of the inflammatory process (Bienvenu et al. 2000). For example in the pathophysiology of septic shock TNFα is a thought to be a principal and early mediator. However, IL-6 has been shown to be a more reliable and sensitive marker, being a final result of the proinflammatory cytokine cascade (Hack et al. 1989).

The maximum urinary excretion of IL-6 was increased markedly compared to values detected after one year. The maximum urinary IL-1β and IL-1Ra excretion measured during infection were low or moderate but also significantly higher than those measured after one year. Further, the excretion of IL-6, IL-1β and IL-1Ra correlated significantly with albumin, IgG and protein excretion.

The half-life of cytokines is very short, usually less than ten minutes. Cytokines can bind to cell and soluble receptors, and the existence of autoantibodies has been documented for TNFα, IL-6 and IL-1 (Bienvenu et al. 2000). There is also some evidence that IL-6 can bind to α2-macroglobulin in serum (Matsuda et al. 1989), but the affinity of this type of binding is rather low (Bienvenu et al. 2000). Plasma proinflammatory cytokines have a low molecular weight (5-50 kD) with either anionic, cationic or neutral charges, and particularly those with positive charges can therefore easily undergo glomerular filtration followed by reabsorption in tubuli and denaturation by proximal tubular cells (Bocci et al. 1993). The urinary excretion of IL-1β and IL-1Ra correlated with the respective plasma levels, suggesting that at least a part of the increases in cytokine excretion is due to filtrated plasma cytokines. On the other hand, the

urinary IL-6 excretion did not correlate with plasma IL-6 levels. Possibly the increases in urinary IL-6 excretion are not entirely due to filtration of increased plasma IL-6, but rather reflect local production of IL-6 in the kidneys during acute NE. Such an idea is further supported by the finding that the fractional clearance of IL-6 did not change during the first three days in hospital, although the fractional clearances of albumin and IgG declined.

There is evidence that the kidney, like other organs, responds to bacterial endotoxin by producing a proinflammatory cytokine response (Kayama et al. 1997). The earliest response observed includes TNFα and IL-1β secretion followed by IL-6, and presumably other inflammatory mediators such as chemokines (Kayama et al. 1997). In inflammatory processes urinary IL-6 is believed to be derived from the kidney, as increased levels do not always coincide with serum concentrations, but do correlate with local inflammatory responses such as proteinuria (Kayama et al. 1997). In this respect, it has been suggested that IL-6 may serve as an indicator of an active inflammatory process within the renal parenchyma.

Shikano and colleagues (2000) showed both serum and urine IL-6 levels to be increased in diabetic patients as diabetic nephropathy progressed. As in this present study, urinary IL-6 levels correlated with urinary albumin excretion, but there was no correlation between serum and urinary IL-6 levels. It was hypothesized that urinary IL-6 may be a good predictor of diabetic nephropathy, since levels correlated with the amount of microalbuminuria. Further, urinary IL-6 levels have been shown to be increased in patients with IgA nephropathy and correlated with mesangial proliferation (Ranieri et al. 1996) and progression of renal disease (Dohi et al. 1991, Harada et al. 2002). In a pediatric population urinary IL-6 and soluble TNF receptor-1 have proved useful markers of reflux-associated renal damage (Ninan et al. 1999). Characteristic of acute kidney allograft rejection is the infiltration of inflammatory cells into the graft, followed by cytokine and adhesion molecule release from the activated cells. Elevated serum and urinary IL-6 levels have been detected in acute allograft rejection (Yoshimura et al. 1991, Waiser et al. 1997), and especially urine IL-6 values have been shown to be sensitive indicators of rejection (Waiser et al. 1997).

The cellular source of urinary IL-6 has not to date been identified. In the kidney, endothelial, mesangial, glomerular and tubular epithelial cells are capable of producing cytokines. In the normal kidney, IL-6 is expressed in the glomerular mesangial area and in the walls of the interstitial arteries, but not in tubules (Fukatsu et al. 1991). IL-6 is, however, expressed in tubular epithelial cells of patients with various forms of renal diseases, and associated with tubular atrophy (Fukatsu et al. 1991). The cellular source of urinary IL-6 in acute NE might be infiltrated cells such as lymphocytes and monocytes. A contribution of renal cells cannot be excluded. Previously, increased expression of TNFα together with TGFβ and PDGF has been detected in the peritubular area of the distal nephron in acute-phase NE kidney biopsies (Temonen et al. 1996). IL-6 was not assessed in that study. Furthermore, in a more recent study, DNA arrays were used to define endothelial cell transcriptional responses elicited by hantaviruses (Geimonen et al. 2002), and IL-6 was found to be included in a group of cytokines which are induced by HFRS-associated HTNV but not with non-pathogenic Prospect Hill virus.

Proinflammatory cytokines are thought to play a role in the pathogenesis of the vascular leakage commonly seen in severe hantaviral diseases. However, a variety of other infectious diseases show elevations in TNFα and other proinflammatory cytokines without profound alterations in capillary permeability. A potential explanation is that the cytokines measured here may be only markers of endothelial pathology rather than its primary cause. Recent studies concerning integrins suggest one possible link between hantavirus endothelial cell interactions and disease (Gavrilovskaya et al. 1999, Mackow and Gavrilovskaya 2001, Gavrilovskaya et al. 2002).

There are some methodological concerns in urinary and plasma cytokine measurements. Urine-derived data depend on urine volume, which is variable especially in acute NE. In this present study cytokine excretion was calculated instead of urinary cytokine levels. Urinary cytokines may also be altered by renal insufficiency. Here, however, only the maximum urinary IL-1β excretion correlated very slightly with serum creatinine concentrations. Further, urinary cytokine results may vary due to circadian variations in excretion. For this reason

timed over-night excretion was measured instead of spot samples. In cytokine measurements, commercial ELISA immunoassays known to be of high specificity and reproducibility were used. The major drawback in immunoassays lies in the fact that they measure a cytokine as an antigen, even though the molecule might be devoid of any biological activity (Bienvenu et al. 2000).

Long-term outcome

Compared to seronegative controls mean GFR values were increased in patients who had suffered from acute PUUV infection 3 to 7 years earlier. This was an unexpected finding, since glomerular function has previously been reported to be either normal or slightly decreased in some patients subsequent to NE (Lähdevirta 1971, Lähdevirta et al. 1978, Elisaf et al. 1993). However, after a closer consideration of individual patient data from a previous Finnish study, 5 out of 20 (25%) patients with endogenous creatinine clearance above 130 ml/min/1.73m^2 could be found (Lähdevirta 1971).

The calculated filtration fraction was also higher in the patients compared to the controls, suggesting an altered balance between the tone of the afferent and efferent glomerular arterioles. This might lead to increased intraglomerular pressure as the mechanism of hyperfiltration. Ala-Houhala and associates (2002) determined GFR by inulin clearance and ERPF by para-aminohippurate clearance in 8 patients with acute NE and in 8 healthy controls. Six patients were followed for 1 year. During the acute phase of NE, significantly reduced GFR and ERPF with high FF were noted in the patients. After 1 year, the GFR of the patients no longer differed from that of controls, but ERPF was still lower and FF higher.

The possibility that hyperfiltration could have been influenced by systemic hypertension was considered. There was, indeed, a slight positive correlation between GFR and mean ambulatory SBP ($r=0.32$, $p=0.040$) in the patient group. Also an analysis of covariance showed that blood pressure had an effect on GFR.

Nonetheless, the difference in GFR between the study groups remained after adjustment for blood pressure.

The patient group as a whole showed higher median urinary protein excretion than the controls. However, the amount of proteinuria in the patients was slight. Twelve out of 45 (27%) patients had urinary protein excretion over 0.22 g/d, with a maximum excretion of 0.38 g/day. The patients more frequently evinced increased urinary excretions of α1-microglobulin than the controls, reflecting alterations in tubular function. The findings of mild tubular defects are in accordance with the results of two previous Finnish follow-up studies (Lähdevirta 1971, Lähdevirta et al. 1978).

There have been occasional reports of an association between previous hantavirus infection and subsequent hypertension (Rubini et al. 1960, Lähdevirta 1971, Lähdevirta et al. 1978, Glass et al. 1990, Kleinknecht and Rollin 1992). The patients in the present series had higher ambulatory SBP than the controls as a group. However, only 7 out of 42 (17%) successful ABP recordings of the patients showed elevated values (Staessen et al. 1994), while other measurements were within normal range. This would suggest that hypertension is not a frequent sequel to NE. The patients also had higher ambulatory heart rates than the controls, this possibly reflecting increased sympathetic activity.

What could then be the mechanism underlying hypertension developing after HFRS? It seems clear that essential hypertension is a complex disease with many causes. It is also well established that the kidney has a key role in the pathogenesis of essential hypertension (Cowley and Roman 1996). Cowley and associates (1995) have proposed a hemodynamic mechanism for the pathogenesis of hypertension, one that involves a reduction in renal medullary blood flow causing local tissue ischemia. Johnson and co-workers (2002) have presented further evidence that renal microvascular disease accompanied by interstitial inflammatory and tubular changes plays a critical role in the genesis of hypertension. They propose that the initiation of renal injury may be induced by any factor which causes renal vasoconstriction. Renal vasoconstriction results in intrarenal ischemia, particularly in the outer medulla and in the cortex adjacent to it. Ischemia of the tubules up-regulates the expression of leukocyte adhesion molecules and induces leukocyte infiltration, which causes local injury. Hypoxia

could also stimulate the generation of cytokines. Arteriolopathy may result when a vasoconstrictive agent stimulates the proliferation of smooth-muscle cells directly or through endothelial-cell activation (Johnson et al. 2002).

Hypothetically, hantavirus infection could act as an initial factor which induces renal vasoconstriction and then results in a process such as that described above. Autopsy studies have shown that the renal medulla, which is particularly susceptible to ischemia, is evidently the most affected part of the kidney in HFRS (Gajdusek 1982). In NE, the most characteristic histological findings include congestion and hemorrhages around the vessels in the outer medulla or corticomedullary junction, with interstitial inflammatory cell infiltrates (Lähdevirta 1971, Collan et al. 1991, Mustonen et al. 1994b). Increased expression of TNFα, TGF-β and PDGF have been detected at the same locations with the inflammatory cell infiltrates, concomitantly with endothelial adhesion molecules (Temonen et al. 1996). The predilection of hantaviruses to infect vascular endothelial cells (Yanagihara and Silverman 1990, Pensiero et al. 1992) may also have a role in the pathogenesis.

In the present series there were a few patients who developed very mild proteinuria and/or hypertension, while the majority did not. It is tempting to assume that residuals after NE are associated with its severity. Such a view was supported by the finding that 5 out of the 9 patients with elevated ambulatory or office blood pressures had suffered from marked renal failure during the acute PUUV infection. On the other hand, neither urinary protein excretion nor overnight α1-microglobulin excretion showed a correlation with the severity of previous NE. There is evidence suggesting that hantavirus infection without acute clinical disease may predispose to the development of hypertension (Glass et al. 1990). In the US survey in question, almost all (14/15) individuals seropositive for SEOV were clinically hypertensive, although only one of them had a clinical history of an HFRS illness (Glass et al. 1990). It is thus possible that a subclinical hantavirus infection may also have chronic consequences.

There are a number of methodological problems concerning this follow-up study. Urinary protein collections and ABP measurements were obtained only once. The weakness relating to single examinations was realized, but the complex study protocol made it unfeasible to carry out more measurements.

Secondly, the single-injection method used in ^{51}CrEDTA clearance studies could give falsely high GFR values due to inaccurate technique. Such systematic errors cannot however explain the difference between the study groups. Values indicating hyperfiltration can be seen in edematous patients and in patients with diabetes mellitus. None of the study subjects had edema or hyperglycemia. Finally, smoking habits and detailed information on alcohol consumption among the study subjects were not registered. It is noteworthy that smoking increases the urinary albumin concentration, even in the range of albumin concentrations below the level of microalbuminuria (Orth 2002). Smoking may also have effects on blood pressure. ABP measurements have documented that parallel with stimulation of the sympathetic system smoking causes a significant, albeit transient (approximately 30 min) increase in blood pressure (Orth 2002).

Three to seven years after NE, the present patients as a group showed higher GFR, FF, ambulatory SBP and more urinary protein excretion than the controls. The study groups were comparable with respect to age, BMI, gender distribution and socio-economic status. Presumably other lifestyle factors not considered here could not account for the differences between the groups. It should be noted that clinically significant alterations in blood pressure and urinary protein excretion were observed only in a minority of patients. Thus, the long-term prognosis of patients with previous NE was favorable in general.

Five patients developed nephrotic syndrome in the convalescent phase of an otherwise typical PUUV infection. The renal biopsy disclosed MCGN in all of them. Transient nephrotic-range proteinuria occurs in 25% of patients with acute hospital-treated NE (Mustonen et al. 1994a), but protein excretion decreases rapidly during the polyuric phase (Lähdevirta 1971, Settergren et al. 1990). In all 5 patients described here, nephrotic-range proteinuria was observed during the convalescent phase (2 to 3 weeks after onset of symptoms typical of acute NE).

The most frequent and long recognized virus-related forms of glomerulonephritis are those associated with hepatitis B virus (HBV) (Glassock 1991). HBV-related glomerulonephritis include membranous, mesangiocapillary and IgA glomerulonephritis. Hepatitis C virus (HCV) is reported to cause MCGN, which is often cryoglobulinemia-related, and also other forms of glomerulonephritis (D'Amico 1998). HIV is closely related to collapsing focal

segmental glomerulosclerosis. The development of glomerular disease in viral infections may depend on various mechanisms. Besides the classical immune complex disease, viral proteins can exert a direct cytopathic effect of the virus on the glomerular cells, and a direct effect on T cells which alters the helper-to-suppressor ratio and affects humoral immunity (Williams 1998). Several findings in the present study speak in favor of an immune complex-mediated disease: the patients had hypocomplementemia and immunoflorescence studies revealed heavy gromerular deposits of C1q or C3. These findings are compatible with activation of the classical pathway often found in type I MCGN.

Most cases of MCGN are idiopathic (Williams 1998). The condition may, however, be observed during the course of many diseases involving chronic antigenemic states, including SLE and hepatitis B and C (with or without cryoglobulinemia), bacterial endocarditis and infected shunts. Further, it has been described to be associated with several infections such as streptococcal and staphylococcal infections, tuberculosis, leprosy, malaria, schistosomiasis, candidiasis, filariasis and mycoplasma infections, as well as with malignancy (especially chronic lymphocytic leukemia) (Williams 1998). The present patients yielded no findings of such diseases. PUUV antigen was not sought in kidney biopsies from the patients. There is no evidence of persistent hantavirus infection, and clinical re-infections with PUUV or other human hantaviruses have never been reported. Viral RNA can easily be detected within the first 9 days of symptomatic PUUV infection, but convalescnt samples (>10 days after onset of symptoms) are consistently PCR-negative (Hörling et al. 1995, Plyusnin et al. 1997a, Plyusnin et al. 1999).

Type I MCGN is defined by the presence of immune deposits in the subendothelial space (capillary wall thickening) and in the mesangium, while type II is characterized by the presence of dense homogenous deposits within the mesangium and in many renal basement membranes. D'Amico and Ferrario (1992) have further characterized six morphological variants of MCGN, and in four of the present patients there were features resembling those of the exudative form of MCGN. This MCGN variant has been reported to be associated with infectious diseases (D'Amico and Ferrario 1992).

In the follow-up examinations, 4 out of 5 patients were in clinical remission, although 2 of them had mild proteinuria and hematuria. In general, the prognosis of MCGN is unfavorable. Spontaneous remission has been reported only in a range of 2 to 20% of patients with MCGN (Williams 1998). There is however some evidence from an Italian study that the prognosis in the exudative variant of MCGN might not be as poor as in some other MCGN variants (D'Amico and Ferrario 1992).

SUMMARY AND CONCLUSIONS

The influence of host genetic factors on the clinical picture of and susceptibility to NE, the association of cytokines on the renal involvement in NE, and the outcome of NE can be summarized as follows:

I The findings pointing to clinically severe NE were strongly associated with the HLA alleles B8 and DR3. Especially the severity of renal failure and the occurrence of shock correlated with this haplotype. The allele frequencies of HLA-B8 and -DR3 in hospitalized patients with NE were increased compared with those among controls. The overrepresentation of these alleles in this material possibly reflects an association between severe NE and the HLA-B8-DR3 haplotype, since it can be assumed that only patients with the most severe disease are hospitalized.

II The patients with TNFα (-308) G/A polymorphism (TNF2) seemed likewise to suffer from clinically severe NE. However, this was probably due to strong linkage disequilibrium with HLA-B8-DR3 haplotype, since no differences in the clinical severity of NE between TNF2-positive/B8-DR3-negative persons and TNF2-negative/B8-DR3-negative persons were noted.

III Hospitalized patients with NE were shown to be more often non-carriers of IL-1Ra allele 2 and IL-1β(-511) allele 2 than PUUV-seronegative blood donors. There were no differences in clinical severity of NE either between IL-1Ra allele 2 carriers and non-carriers, between IL-1β(-511) allele 2 carriers and non-carriers or between IL-1Ra allele 2/IL-1β (-511) allele 2 non-carriers and carriers.

IV Especially urinary IL-6 excretion was markedly increased in patients with acute-phase NE compared with values detected after one year. Urinary

IL-6 excretion showed a significant positive correlation with urinary albumin and protein excretion but none with plasma IL-6.

V Three to seven years after NE, the patients as a group showed more urinary protein and α1-microglobulin excretion and higher GFR and FF, mean ambulatory SBP and heart rate than the seronegative controls.

VI As an uncommon phenomenon, MCGN might develop in the convalescent phase of NE.

In conclusion, the conserved haplotype containing HLA-B8, -DR3 and TNFα(-308) 2 alleles .is associated with a severe clinical course of NE. The TNF2 allele may not be an independent risk factor for severe NE but a passive component in the extended HLA haplotype. The findings suggest that the urinary cytokines, notably IL-6, are at least partly locally produced in the kidneys by infiltrating monocytes/macrophages and/or by renal cells. Since cytokines might not be more than markers of active inflammation, the possible contribution of increased urinary IL-6 excretion to the transient massive proteinuria seen in patients with acute NE awaits further investigations. The prognosis of NE is favorable. Nevertheless, the infection may cause mild tubular lesions and hypertension in some patients. Chronic glomerulonephritis after PUUV infection is without doubt a rare phenomenon. Further follow-up with larger materials is necessary to establish the final outcome of NE.

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