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Long-Lived Memory T Lymphocyte Responses Following Hantavirus Infection: a Dissertation

Heather Lin Van Epps
University of Massachusetts Medical School

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LONG-LIVED MEMORY T LYMPHOCYTE RESPONSES FOLLOWING HANTA VIRUS INFECTION

A Dissertation Presented

By

Heather Lin Van Epps

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
Program in Immunology and Virology

July 18, 2001
LONG-LIVED MEMORY T LYMPHOCYTE RESPONSES FOLLOWING HANTA VIRUS INFECTION

A Dissertation Presented By
Heather L. Van Epps

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Dr. Thomas B. Miller, Dean of the Graduate School of Biomedical Sciences
Program in Immunology and Virology
July 18, 2001
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Parts of this dissertation have been presented in the following publications:


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First and foremost, my sincere thanks to my mentor, Dr. Ennis, whose guidance and encouragement made graduate school an exciting and gratifying experience. Your commitment to learning, enthusiasm for immunology, and unwavering support for the students have been truly inspiration. My thanks to the CIDVR crew whose help, support and company sustained (and entertained) me every day. To my predecessors, Susan, Anuja, Julie and Vicki - you're a tough act to follow! Thanks for the friendship and encouragement - you each brought something unique to the lab and to science, and I have learned from each of you. Many thanks also to Alan Rothman for his advice, his incredible brain, and his amazing ability to find time for every student. Journal club wouldn't have been the same without you!

Thanks to my family whose unconditional support and love have guided me through every step of my life. Thank you for your genuine interest in all my endeavors, and for teaching me the value of hard work and commitment. Finally, a huge thanks to my friends, old and new, who have always been a phone call away, and without whom I would be lost.
ABSTRACT

Hantaviruses are members of the virus family Bunyaviridae that cause two potentially life-threatening diseases in humans: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). HFRS is caused by Old World hantaviruses that are endemic in many Asian and European countries. Infections with Old World hantaviruses can range in severity from asymptomatic to moderate or severe, depending primarily on the infecting serotype of virus. HPS is caused by New World hantaviruses in North and South America. New World hantaviruses are rarely asymptomatic and are severe in the majority of cases. These syndromes are distinct from one another in the primary target organ of virus infection (kidney vs. lung), but have important clinical features in common, including fever, thrombocytopenia, and a capillary leak syndrome. These common clinical manifestations suggest that the underlying mechanisms of disease may be similar in the two syndromes.

The precise mechanisms of pathogenesis of HFRS and HPS are poorly characterized, but may be mediated in part by immunopathology. Hantaviruses are able to establish infections in many human cell types, including primary human endothelial cells, without having any cytopathic effect on these cells. Human infections with hantavirus result in a robust activation of the humoral and cellular immune response, and we hypothesize that these immune responses contribute to the pathology of disease. Evidence for the activation of T lymphocytes, and their potential involvement in immunopathology, includes increases in the number of circulating, activated CD8+ T
cells during HFRS, the presence of lymphocytic infiltrates (predominantly CD8+ T cells) in kidney biopsies from patients with acute HFRS, and associations between certain HLA haplotype and disease severity following hantavirus infection. This thesis is the first examination of human T lymphocyte responses that are generated during HFRS. Initially, we studied memory T cell responses in scientists who were sub-clinically infected with Hantaan virus (HTNV), the prototype hantavirus. We later investigated memory T cell responses in healthy Finnish adults who had HFRS caused by Puumala virus (PUUV), a hantavirus endemic primarily in Scandinavia.

At the onset of these studies, there was no available information on human T lymphocyte responses to Old World hantaviruses. Virus-specific CD8+ and CD4+ human T cell lines had been isolated from patients with acute HPS caused by Sin Nombre virus (SNV) infection. In that study, conducted in our laboratory, several human T cell epitopes on the nucleocapsid (N) protein and G2 envelope glycoprotein of SNV were identified and characterized. We decided to perform similar analyses on PBMC from donors who had been infected with HTNV and PUUV, in order to determine the specificity and diversity of the T cell response to Old World hantaviruses.

The initial study of three donors who had sub-clinical infections with HTNV demonstrated that virus-specific T cell responses could be detected in all the donors following in vitro stimulation of PBMC with inactivated virus. In two of the donors, the virus-specific cytolytic T cells (CTL) recognized the HTNV N protein, and in the third donor the virus-specific CTLs recognized the HTNV G1 glycoprotein. Isolation and characterization of virus-specific T cells from two donors resulted in the identification of
two CD8\(^+\) T cell epitopes on the HTNV N protein, which were restricted by either HLA A1 or B51. These CTL lines included both HTNV-specific (HLA B51-restricted) and serotype-cross reactive (HLA A1 restricted) lines. In one subject, these virus-specific T cell responses were detectable in IFN-\(\gamma\) ELISPOT assays following peptide stimulation, and in bulk cultures after short-term stimulation with inactivated HTNV. These results indicated that the CD8\(^+\) CTL responses of humans after sub-clinical infection with HTNV were readily detectable and were directed against a limited number of viral proteins and epitopes. In addition, sub-clinical infection resulted in the generation of both virus-specific and cross-reactive CTL responses.

We reasoned that hantavirus infections that lead to clinical illness may result in the generation of more robust and/or diverse virus-specific T cell responses than in sub-clinical infections. To address this question, we studied the memory CD8\(^+\) T cell responses in a group of healthy adults from Finland who had HFRS caused by PUUV infection between the years 1984 and 1995. We detected virus-specific CTL in the bulk cultures of seven of eleven immune individuals tested following stimulation with infectious virus. The PUUV proteins N, G1 and G2 were recognized by CTLs in six, five, and two donors respectively. Extensive cloning of T cells from two donors resulted in the isolation of sixty-three virus-specific CTL lines, the majority of which (61/63) were specific for the PUUV N protein. Six novel CD8\(^+\) CTL epitopes and one CD4\(^+\) CTL epitope were identified on the N protein, all of which clustered in the center of the protein between amino acids 173 and 251. The CTL lines specific for these epitopes were restricted by a variety of HLA alleles including A2, A28, B7 and B8, and were
primarily serotype specific when tested against target cells expressing HTNV or SNV N protein. IFN-γ ELISPOT analysis using the defined epitopes to stimulated PBMC, revealed high frequencies of circulating N-specific CD8+ T cells in eight of thirteen individuals tested. Finally, T cell receptor (TCR) Vβ analysis of CTL clones specific for one epitope (N_{204-12}) demonstrated that cells in this population expressed up to five different Vβ chains. These results demonstrated that the PUUV N protein may be the dominant target of the CTL response, that the N-specific CD8+ CTL responses are diverse, heterogeneous, and primarily serotype specific, and that virus-specific memory CD8+ T cells can persist at high levels for up to 15 years after the primary infection.

In order to understand the pathology of HFRS and HPS, we must be able to assess the contribution of various factors that could potentially contribute to disease. The virus burden in the infected individual is likely to be an important factor in the severity of the resulting disease. Quantitative RT-PCR analysis of plasma samples from acute HPS patients demonstrated that a higher virus burden (as reflected by viral RNA copy number) is associated with more severe HPS. In order to perform similar analyses in patients with HFRS caused by PUUV, we established a quantitative RT-PCR assay for the detection of PUUV S segment RNA in patient plasma. The design and optimization of the PUUV-specific RT-PCR is described in this report. This assay will allow us to measure the virus burden in patients and compare these data with levels of T cell activation and with parameters of disease severity. In this way, we hope to gain an understanding of the kinetics and magnitude of both the virus burden and virus-specific T cell response during the acute illness.
This thesis provides the first description of human virus-specific T cell responses to HTNV and PUUV. These data shed light on the nature of the CD8$^+$ T cell responses that are generated following natural infections with PUUV and sub-clinical infections with HTNV. The studies of memory CD8$^+$ T cell responses to PUUV, and the development of a PUUV-specific quantitative RT-PCR assay, establish the framework for future studies of the immunopathology of acute HFRS. Quantitative analysis of both virus burden and T cell responses during acute illness will provide insight into their relative contributions to the pathology of disease.
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<td>APC</td>
<td>antigen presenting cell</td>
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<td>E/T</td>
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<td>MHC</td>
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<td>N</td>
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<td>TCR</td>
<td>T cell receptor</td>
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<td>tumor necrosis factor receptor</td>
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<td>vRNA</td>
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CHAPTER I
INTRODUCTION

The genus *Hantavirus* of the virus family *Bunyaviridae* is comprised of more than twenty viruses, approximately half of which are known to cause human disease (1, 2). Hantaviruses are rodent-borne viruses that are transmitted to humans by inhalation of aerosolized virus particles that are shed in rodent excreta. This characteristic renders hantaviruses distinct from the other four genera of the family *Bunyaviridae*, which are transmitted via arthropod vectors (3). Hantavirus infection in humans can result in the development of two distinct and potentially life-threatening diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) (2, 4). Some examples of different hantaviruses, their rodent hosts and resulting diseases are shown in Table I-1.

HFRS is caused by Old World hantaviruses endemic in many Asian and European countries including China, Japan, Korea, Russia, Finland, Sweden, Hungary, France, Belgium, Germany, Slovakia and Slovenia. Approximately 100,000-200,000 clinical cases of HFRS are reported annually, more than half of which occur in China (1, 5). Human disease resulting from Old World hantavirus infections can range from a subclinical or mild illness to a moderate or severe syndrome, depending primarily on the infecting strain of virus. The most severe cases of HFRS are characterized by fever, hemorrhage and renal failure and are fatal in 5-15% of cases (4, 5). Hantaan virus (HTNV), the prototype hantavirus, was first isolated in 1978. HTNV, which is carried by the striped field mouse (*Apodemus agrarius*), is endemic primarily in Asia and causes a severe form of HFRS (6). Other hantaviruses that cause HFRS include Dobrava virus (DOBV), carried by the yellow-necked field mouse (*Apodemus flavicollis*) in the Balkans; Seoul virus (SEOV), carried by the gray rat (*Rattus norvegicus*) worldwide; and Puumala virus (PUUV), carried by the bank vole (*Clethrionomys*
glareolus) in Scandinavia, Western Europe and the Balkans. DOBV and SEOV are associated with moderate to severe forms of HFRS, while PUUV causes a milder disease (also called nephropathica epidemica or NE).

Recent outbreaks of illness in the Southwestern United States and South America have resulted in the isolation of a newly recognized group of hantaviruses (e.g. Sin Nombre (SNV) and Andes (ANDV) viruses) that cause the fatal pulmonary disease HPS (7-9). HPS causes severe disease in most cases and has a case fatality rate of 30-50%.

HFRS and HPS are distinct in the predominant target organ of virus infection (kidney vs. lung) but have important clinical features in common including fever, thrombocytopenia and a capillary leak syndrome (2, 10). The common clinical manifestations suggest that the underlying mechanism of disease may be similar in the two syndromes.
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A. HANTAVIRUS VIROLOGY

Like all viruses in the genus *Bunyavirus*, hantaviruses are enveloped viruses with a diameter of 90-110 nm and a segmented, single-stranded RNA genome. The virus genome consists of three strands of negative-sense RNA designated S (small; 1600-2100 nt), M (medium; ~3700 nt) and L (large; ~6500 nt) (11). The hantavirus RNA segments are flanked by short, complementary regions of 9-11 nucleotides which may hybridize to form panhandle structures, similar to those seen in electron micrographs of other members of the bunyavirus genus (11). Each RNA segment contains a single long open reading frame and encodes a single protein product (3, 12).

The S segment encodes the viral nucleocapsid (N) protein, a 45K protein that associates with the viral RNA in the mature virion (13), and is thought to play a role in replication and encapsidation of the viral RNA (14, 15). Cross-linking and electrophoresis mobility shift studies utilizing deletion RNA derivatives and synthetic oligoribonucleotides have demonstrated that the N protein binds to the 5' untranslated region of the S segment (16). The N proteins from the different hantaviruses share an overall amino acid homology of approximately 50%, although specific regions of the protein are more or less highly conserved (amino acids 340-433 are 85% homologous; amino acids 240-310 are 11% homologous). N proteins of closely related viruses such as HTNV and SEOV, or SNV and PUUV, are more highly conserved, sharing approximately 80-85% and 70-71% identity at the amino acid level, respectively (12, 17).

The M RNA segment encodes the precursor to the envelope glycoproteins, which is co-translationally cleaved into the mature G1 and G2 envelope glycoproteins (18, 19). G1 and G2 are type I glycoproteins that dimerize in the ER and are subsequently transported to the Golgi where virus assembly and budding occur (3). The glycoproteins are thought to bind
the virus receptors on target cells, which have recently been identified as members of the β3 integrin family of proteins (20, 21). Comparisons of the nucleotide sequences of the M segment from various hantaviruses shows that the M and S segments share a comparable degree of homology. This suggests that the S and M segments have evolved at approximately the same rate (12). This is in contrast to other RNA viruses, such as influenza, in which the external proteins mutate at a significantly faster rate than the internal proteins (22). The amino acid sequence identity of the G1 and G2 proteins ranges from 50-80% between different hantaviruses. The position of several conserved cysteine residues within the protein sequences suggests that the overall structure of the glycoproteins is essentially the same among all hantaviruses (19).

The L segment encodes the viral RNA-dependent RNA polymerase (23). The L protein is believed to carry out both transcription and replication of the viral genome, as well as the transcription of complementary and messenger RNAs. The L segment has a slightly higher level of nucleotide sequence homology between the hantaviruses than the S or M segments, which is likely to reflect a more stringent requirement for conservation of structure and/or function for the replicative enzyme than for the nucleocapsid or the envelope glycoproteins (12).

Hantaviruses are carried in nature by rodents belonging to the murinae, sigmodontinae and arvicolinae subfamilies of the rodent family Muridae (2, 24). Each hantavirus is carried by a distinct rodent species and phylogenetic analysis of viral nucleotide sequences suggests that hantaviruses have co-evolved with their rodent hosts for thousands of years. Hence, hantaviruses that are carried by the same subfamily of rodent (e.g. HTNV, DOBV and SEOV) are more genetically and antigenically similar to one another than those carried by different
rodent subfamilies. A phylogenetic tree of the different hantaviruses (based on the nucleotide sequences of the S segment) is shown in Figure I-1.
Figure I-1. Phylogeny of Hantaviruses: Based on Sequence of S Segment (Subfamily, Hantavirus, Location, Host)

**Figure Description:**
- **HTN Korea** - *Apodemus agrarius*
- **DOB Slovenia** - *Apodemus flavicollis*
- **SEO Japan** - *Rattus norvegicus*
- **PUU Finland** - *Clethrionomys glareolus*
- **PUU Russia** - *Clethrionomys glareolus*
- **PUU Sweden** - *Clethrionomys glareolus*
- **PUU Belgium** - *Clethrionomys glareolus*
- **TOP Russia** - *Lemmus sibiricus*
- **KBR Russia** - *Microtus fortis*
- **TUL Russia** - *Microtus arvalis*
- **TUL Czech** - *Microtus arvalis*
- **TUL Slovakia** - *Microtus arvalis*
- **PH New York** - *Microtus pennsylvanicus*
- **PH Maryland** - *Microtus pennsylvanicus*
- **ISLA California** - *Microtus californicus*
- **MULE Southern US** - *Sigmodon hispidus (texensis)*
- **BCC Florida** - *Sigmodon hispidus*
- **BAY Southeastern US** - *Oryzomys palustris*
- **ANDES Argentina & Chile** - *Oligoryzomys longicaudatus*
- **LEC Argentina** - *Oligoryzomys flavescens*
- **RIOM Bolivia & Peru** - *Oligoryzomys microtis*
- **LN Paraguay & Bolivia** - *Calomys laucha*
- **SN New Mexico** - *Peromyscus maniculatus*
- **SN California** - *Peromyscus maniculatus*
- **NY New York** - *Peromyscus leucopus*
- **NY Rhode Island** - *Peromyscus leucopus*
- **MON West Virginia** - *Peromyscus maniculatus*
- **ELMC Western US & Mexico** - *Reithrodontomys megalotis*
- **RIOS Costa Rica** - *Reithrodontomys mexicanus*
1. Virus transmission and tissue localization in natural rodent hosts.

Hantaviruses are maintained in nature via cyclical transmission between rodents. Infection of the natural rodent hosts appears to be lifelong and asymptomatic (1, 25). Infection between mice is thought to occur in an age-dependent, horizontal fashion and this has been demonstrated in both wild (25, 26) and experimental settings (27-29). Transmission may occur by bites inflicted during aggressive encounters or via inhalation of contaminated aerosols from urine, feces and saliva (25, 30). Observations of PUUV infection among wild bank voles also suggest that transmission among rodents may depend on the sexual maturity of the rodents, particularly for males (25). In this study, seroconversion occurred more frequently during the reproductive season of the vole and in sexually mature males. This may reflect increased aggressive behavior, communal nesting habits and increased mobility of the rodents, all of which generally occur at the onset of sexual maturity.

In some species of rodents that carry New World hantaviruses, such as *Sigmodon hispidus* (31), *Peromyscus maniculatus* (32) and *Reithrodontomys megalotis* (2), male mice are more commonly infected than females. However, in other species, like *C. glareolus*, sex-related differences in seroprevalence are uncommon. Young rodents possess protective maternal antibodies against hantavirus, which disappear by 35-45 days of age, after which the rodents become susceptible to hantavirus infection (25, 27, 33).

Infection results in extensive dissemination of virus in the rodent. There is a transient viremia during which time the animals are capable of transmitting virus in a horizontal fashion (25, 30, 31, 34, 35). Evidence from natural and experimentally infected animals indicates that virus replication and shedding reach a peak during the first month of infection and decrease thereafter (25, 31), although the duration and intensity of infectious virus
shedding varies with the host species and infecting virus. A study of wild-caught bank voles demonstrated that PUUV-specific antibodies, virus antigen, infectious virus and the ability to transmit virus to cage-mates lasted for at least 15 months after infection (25). In that study, antibodies persisted throughout this time period, while the intensity of virus reproduction, the frequency of horizontal transmission and the accumulation of PUUV antigen decreased over time (25). Viral antigen and infectious virus can be detected in various organs of the infected rodent including heart, lung, liver, spleen, kidney and brown fat as well as in urine, feces and saliva (30, 31, 34, 36). Following experimental infection of P. maniculatus, the natural reservoir for SNV, the highest titers of viral RNA and levels of viral antigen were detected in the heart, lung and brown fat (34). Studies examining the specific types of cells infected by hantaviruses in mice are sparse. However, the aforementioned study of SNV infection in P. maniculatus found that the cells infected with the virus had the characteristic morphology of endothelial cells (34). Following natural infections with PUUV in bank voles, strong cytoplasmic staining of macrophage-like cells in the lungs was observed (25). These data suggest that endothelial cells and macrophages are likely targets of hantavirus infection in rodent hosts, and studies of experimental infection in rodents support this finding (29, 37).

2. **Immune responses in natural rodent hosts.**

Infected rodents mount both humoral and cellular immune responses to hantaviruses. Most of the evidence for this is derived from studies in which wild-caught rodents are experimentally infected with their native hantavirus species (30, 34). Experimental infections of wild bank voles (C. glareolus) infected with PUUV showed that virus-specific antibody responses appeared 18 days after intra-muscular (i.m.) inoculation and were maximal 4-5 weeks post-inoculation (29). Antibody levels persisted at moderate levels for the duration of that study (270 days). Similar kinetics of antibody generation were observed following
experimental infection of *S. hispidus* with Black Creek Canal virus (BCCV) and infection of *P. maniculatus* with SNV (31, 34). As the rodents generate an immune response, the amount of viral antigen decreases, but virus is not cleared and can persist in the lung and other tissues for at least one year after infection (25, 30, 35). To date, no studies of T cell responses to hantavirus infection have been performed in the natural host species, but some groups have observed that infected organs often contain monocytic infiltrates, which may include activated T lymphocytes (35, 38).

No signs of clinical illness were apparent in the rodent hosts in any of these studies (25, 31, 34). Most studies also revealed a lack of gross histopathological changes in infected organs of mice (29, 30, 39). However, studies of *Peromyscus maniculatus* and *P. leucopus*, which carry SVV and New York virus (NYV) respectively, have shown evidence of septal edema in lung tissue and mononuclear cell infiltrates in the liver (35, 38). The authors of these two studies suggested that such changes in tissue morphology are similar to those associated with SNV infections in humans. However, the general consensus is that hantavirus infection in the natural rodent hosts is completely asymptomatic (27, 40).

3. Transmission of hantavirus to humans.

Hantavirus infection of humans is considered to be an accidental, “spill-over” infection that occurs via inhalation of aerosolized virus particles shed in rodent excreta. Outbreaks of hantavirus disease in humans have been associated with increases in rodent populations, which fluctuate with season, food supply and weather patterns (40, 41). Human infections are also associated with activities that increase contact between rodents and humans (2, 42). Spring and summer outbreaks of HFRS in agricultural settings in Asia and Europe have been associated with contact between humans and field rodents related to the planting and harvesting of crops (24). In Scandinavian countries, the incidence of HFRS peaks in the
fall and winter months, which correlates with the movement of rodents into man-made structures such as houses and barns (43). Occupational risk of hantavirus infection is high among animal trappers, forestry workers, farmers and military personnel due to an increased likelihood of contact with rodents (44).

Outbreaks of human hantavirus infection have also occurred in laboratories in which scientists were working with hantaviruses or handling infected laboratory rats. Laboratory infections have been reported in Japan, Korea, Belgium, England and France (4, 45-48). In one laboratory outbreak in Korea, the infections occurred only during the months of November through April. The authors of that report suggested that factors such as limited air circulation and lower humidity in the animal rooms during the winter months may have increased the probability of virus aerosolization and transmission (45).

Human-to-human transmission is not thought to occur with most hantaviruses (42). However, there have been two reports of human-to-human transmission of ANDV in Argentina (42, 49), in which healthcare workers and friends of HPS patients were infected with genetically identical viruses. Many of the individuals who contracted HPS following patient contact had never been to the endemic region before and had no evident contact with rodents (49), suggesting that transmission occurred through contact with infected humans.


Antibodies to hantaviruses can be detected in humans and rodents throughout the world with seroprevalence rates varying widely, depending on the geographic region and virus species in question. Seroepidemiology studies in the most endemic regions of Norway reported that up to 30% of individuals over 60 years of age were hantavirus seropositive (50, 51). A similar study of 1,800 individuals in an endemic area in rural China reported that 12% of the population was hantavirus seropositive. Seropositive individuals in that study ranged in
age from 2-79 years, and antibody prevalence increased with age (52). In the Philippines, the overall seroprevalence rate was approximately 6%, with little variation between rural, urban and urban poor areas (53). In other non-endemic areas, the reported seroprevalence rates were much lower than in endemic areas, often 1% or less (54). Comparisons of seroprevalence rates and hospitalized cases of HFRS suggest that there are many mild or asymptomatic hantavirus infections in Europe and Asia. The ratio of virus infections to hospitalized illness ranges from approximately 15:1 for PUUV infections to 5:1 for HTNV infections (52, 55).

Serological evidence of past infection with Sin Nombre-like hantaviruses was demonstrated in a study of approximately 200 Indians living in South America. In that study, 17% of Indians living in Western Paraguay and over 40% of Indians living in northern Argentina were antibody-positive, with seroprevalence increasing with age in both populations (56). Studies of these populations are of particular interest since clinically apparent disease appears to be rare in these regions. This observation might suggest that a less pathogenic hantavirus circulates in this area, or that native American populations are genetically resistant to disease. In another study, 23 HPS cases were reported in the Filadelfia area of Western Paraguay with a low case fatality rate of 17% (57). Interestingly, the majority of clinical cases in this region occurred among the Mennonites, who are not indigenous to the area, whereas very few cases were reported among the native Indians. Again, this suggests that the indigenous populations may be more resistant to severe illness caused by hantavirus infection. Alternatively, the authors of that study proposed that the native Indian population may experience greater exposure to the virus due to poor living conditions and/or that the Mennonites may be more likely to report illnesses. This low mortality rate is in stark contrast to Sin Nombre virus infections in the southwestern U.S., which have an overall case fatality rate of approximately 50%.
Rodents throughout the world have been shown to harbor infections with various hantaviruses. Serological analysis of rodents trapped in endemic areas of China and Scandinavia showed that 30-40% of rodent species known to harbor hantaviruses had measurable virus-specific antibodies (52, 55, 58). In Taiwan, where Seoul virus appears to be the only hantavirus in circulation, seroprevalence rates ranged from 5% in rural regions to 20% in international seaports, where the *R. rattus* and *R. norvegicus* carrier species are most prevalent (59). A study conducted in Slovenia reported a 20% seropositivity rate among *Apodemus* species trapped at five study sites over a six year period (60). Sequence analysis of M segment PCR products amplified from the kidney tissues of seropositive rodents in that study revealed that two distinct lineages of DOBV co-circulate in the area.

Several studies have reported seasonal and yearly variations in seroprevalence rates. A study of wild-caught red bank voles (*C. glareolus*) in Belgium reported a high seroprevalence (47.7%) in the spring of 1999, a year associated with a marked increase in rodent density (26). This increase in rodent seroprevalence was also associated with increased disease in humans (61). However, in earlier years much lower seroprevalence rates (~6%) were observed.

Recent serologic studies have been performed on rodents in the Americas. Serologic surveys of rodents in California and Nevada demonstrated that approximately 12% of *P. maniculatus* tested had antibodies to SNV (62, 63). A large study examining hantavirus seroprevalence among rodents in U.S. National Parks demonstrated that rodents throughout the U.S. are antibody-positive for SNV, with the overall prevalence rate ranging from 2% (*Peromyscus leucopus*) to 33% (*Reithrodontomys megalotis*) in different rodent species (32). Interestingly, the seroprevalence among *Peromyscus* species (*P. maniculatus and P. leucopus*) was highest in the northeast, where few cases of HPS have been identified. These data demonstrate the potential for human infection with hantaviruses throughout the United States.
C. CLINICAL DISEASE AND DIAGNOSIS IN HUMANS

As noted earlier, hantaviruses can cause two distinct and severe syndromes in humans: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). The outcome of infection depends primarily on the infecting strain of hantavirus. In general, hantaviruses that are endemic in Asia and Europe primarily target the kidney and cause HFRS, while hantaviruses endemic in North and South America target the lung and cause HPS. The genetic basis for the distinct tropism between the different hantaviruses has not been established. In recent years, detailed clinical analysis of patients with acute hantaviral infections has revealed that many of the ‘classical’ symptoms of HFRS are seen in some HPS cases, and vice versa (1).

1. HFRS

HFRS can appear as a mild, moderate or severe disease, depending primarily on the infecting strain of virus. HTNV and DOBV are known to cause the most severe forms of HFRS, while PUUV causes a less severe form of disease, often referred to as nephropathia epidemica (NE). Mortality rates range from less than 1% for HFRS caused by PUUV to 5-15% for HFRS caused by HTNV (24, 64). The genetic differences among the viruses that contribute to the differences in pathogenicity have yet to be elucidated.

It has been estimated that approximately 30-40% of individuals infected with HTNV will experience a mild clinical course of disease, 50-60% will exhibit a moderate course and 20-30% will experience severe forms of HFRS (4, 58). Severe HFRS typically occurs in four overlapping phases: febrile, hypotensive (shock), oliguric and diuretic (2, 65, 66). However,
it is not uncommon, especially in mild and moderately severe cases, for some of the characteristic phases to be inapparent and the boundaries between phases blurred.

Disease onset follows an incubation period of 2-3 weeks and is characterized by the rapid appearance of non-specific, flu-like symptoms including high fever, chills, myalgia, abdominal and lumbar pain, and malaise (58, 67). During the febrile phase, characteristic hemorrhagic manifestations appear including conjunctival injection, face and neck flushing, petechiae of the skin and soft palate, and retroperitoneal edema (4, 58, 66, 67). These symptoms indicate a generalized alteration in the function of the small blood vessels (68). These bleeding manifestations are commonly observed in severe HFRS, but are rare in milder infections such as those caused by PUUV (67). Most of the clinical features of HFRS that develop during and after the febrile phase are thought to be a result of general capillary dysfunction (68, 69).

Defervescence marks the beginning of the hypotensive (shock) phase, which can last from a few hours to several days. The degree of hypotension can range from mild decreases in blood pressure to profound shock and circulatory collapse, which is attributable to vascular leak (66). Common symptoms observed during this phase of HFRS include tachycardia, restlessness, nausea and vomiting. Typical laboratory abnormalities include proteinuria, thrombocytopenia and hemoconcentration (64). Thrombocytopenia and hemoconcentration are hallmarks of capillary leakage, which are characteristic of HFRS (and HPS) (70). Capillary hemorrhages appear to be most prominent during this phase of the syndrome and predominantly affect the renal medulla, right atrium and gastrointestinal submucosa (66). In addition to the cardiac hemorrhage that is frequently observed during HFRS, there is also a characteristic mononuclear cell infiltration of the edematous layer immediately beneath the endocardial endometrium (66). Hemorrhages of the renal medulla rarely result from the rupture or loss of continuity of vessels, but are instead thought to be the result of vascular
engorgement and diapedesis of red blood cells (66). Approximately one-third of deaths occur during this phase due to irreversible circulatory shock that results from massive fluid loss. Indeed, most fatal cases show evidence of moderate to severe retroperitoneal edema upon autopsy (64, 66, 69, 71).

During the oliguric phase, blood pressure begins to normalize, and some patients experience transient hypertension due to constriction of capillaries and reabsorption of fluid (66, 68). Hemoconcentration and decreased blood volume are generally reversed very rapidly upon recovery from hypotension. The degree of renal failure that develops is variable from patient to patient, but can be severe enough to require dialysis (2, 5). Histological examination of renal tissue from fatal cases of HFRS showed evidence of tubular necrosis in most cases (66).

Bleeding manifestations are apparent during the oliguric phase in approximately 1/3 of severe cases, and include conjunctival hemorrhage, gastrointestinal bleeding, ecchymosis (bruising), epistaxis (nose bleeds) and, in rare cases, cerebral hemorrhage (4, 67). Central nervous system involvement and pulmonary edema have also been observed at this phase in the most severe forms of disease (4). Fifty percent of fatalities occur during the oliguric phase, primarily due to hypervolemia, renal failure, pulmonary edema or a combination thereof.

Diuresis marks the beginning of convalescence, which may last for several days or weeks. The diuretic phase is characterized by polyuria (3-6 L/day), improved renal function and decreased proteinuria. Since non-fatal cases mostly result in complete recovery, the vascular changes observed during HFRS appear to be completely reversible. Complete recovery is observed in the majority of cases, but there have been some reports of long-term renal abnormalities following the resolution of HFRS (72-74).
2. HPS

Hantavirus pulmonary syndrome is a severe illness in the majority of cases. Asymptomatic infection with hantaviruses that cause HPS is not thought to be common, although there have been some observations of individuals who were antibody-positive but did not experience clinical disease (57). The incubation period of viruses causing HPS is thought to be approximately 7 to 14 days, slightly shorter than for viruses causing HFRS.

HPS is characterized by an initial non-specific febrile period that is similar to that observed in HFRS. However, in HPS the febrile phase is followed by the rapid development of severe pulmonary edema and respiratory failure, signaled by severe shortness of breath (5, 75). Chest radiographs show evidence of pulmonary edema, usually within hours after the first respiratory symptoms are evident. Supplemental oxygen is required in the majority of cases, and some patients require intubation and mechanical ventilation.

Death can be extremely rapid in HPS, and is generally attributed to shock and myocardial dysfunction (75). Autopsies of 14 fatal HPS cases revealed bilateral pleural effusions in all patients and interstitial mononuclear cell infiltrates in the majority of patients (75). Recovery from HPS appears to be equally rapid and was until recently thought to be complete. However, Goade and colleagues now report that many patients experience significant, long-term sequelae after HPS (76). In a follow-up study of 32 patients who had HPS caused by SNV or Bayou virus (BAYV), the majority of patients had long-term somatic and physical complaints including fatigue (89%), decreased exercise tolerance (89%), shortness of breath (82%), myalgias (82%) and short-term memory loss (55%). In addition, 87% of patients demonstrated mild to moderate changes in pulmonary function, with the most common findings including significantly decreased small airway flow, increased residual volume and decreased oxygen diffusion capacity. Three patients in that study also
experienced proteinuria during early convalescence and four others developed proteinuria 2 to 3 years post illness (76).

3. Antibody responses and diagnosis

The diagnosis of hantavirus infection is most often based on serology. Hantavirus infection induces high levels of IgM antibodies specific for the N, G1 and G2 proteins that are detectable in serum within the first week of illness. The IgM response to hantavirus N antigens is detectable early after the onset of disease (day 1-5) and remains detectable for 2-3 months (77-81). The IgG response to the PUUV N antigen is detectable early in acute illness (1-5 days after onset of disease), while the IgG response to the PUUV glycoproteins is first detectable in the early convalescent phase (16-30 days after disease onset) (82). In contrast, the SNV glycoprotein-specific IgG response is detectable early in the course of the acute illness (81). The levels of both PUUV N- and glycoprotein-specific IgG were considerably increased 2 years after the disease onset, as compared to levels measure in the early convalescent phase. Hantavirus-specific IgG antibodies are detectable for long periods of time after the resolution of HFRS (80, 83). Following PUUV infections, IgG responses against the three PUUV antigens remain high several decades after the acute infection (78, 82).

The detection of IgM antibodies is the basis for serological diagnosis of acute hantavirus infection and the differentiation between active and past infections. Indirect immunofluorescence techniques using native virus grown in Vero E6 cells have been used historically to detect virus-specific IgM and IgG. More recently, enzyme immunoassays using both native and recombinant S segment antigens have been developed in various forms including a μ capture assay for sensitive detection of PUU virus-specific IgM, which
facilitates early diagnosis (77, 84). Since the antibody responses during acute HFRS are usually highly cross-reactive between hantaviruses (85, 86), diagnosis of the infecting virus strain requires neutralization assays using convalescent sera or RT-PCR amplification and sequencing of viral RNA. Newly developed chromatographic assays using highly purified recombinant antigens appear promising as a tool for rapid diagnosis (87). RT-PCR analysis has also been used to detect viral RNA in tissues and plasma (88-91).

D. EXPERIMENTAL INFECTIONS IN LABORATORY RODENTS

Definition of the mechanisms involved in the pathophysiology of hantavirus infection has been hampered by the lack of a suitable animal model that recapitulates the human disease. Many studies of hantavirus infections in rodents have been carried out, with the outcome depending on several parameters including the species and age of the rodent, route of inoculation and strain of virus. Rodents that are susceptible to lethal infection die of a characteristic meningoencephalitis, rather than a hemorrhagic syndrome affecting the kidneys or lungs. While these studies may therefore have limited applicability to human infections, they have demonstrated a role for T lymphocytes in both the clearance of the virus as well as in pathology of infection (92, 93), a phenomenon that we postulate to occur during human infection.

Studies using immunocompetent strains of laboratory mice, including BALB/c, CD-1 and outbred ICR mice, have demonstrated an age-dependent resistance to infection with a lab-adapted strain of HTN virus (76-118). Mice less than two weeks of age infected intracranially (i.c.) or intraperitoneally (i.p.) developed clinical disease characterized by weight loss, ruffled fur, hunched posture, limb paralysis and eventually convulsions and sudden death due to viral meningoencephalitis (37, 94-96). Virus antigen in those studies was detected in brain, liver,
lung, heart, thymus spleen, pancreas and kidney tissues of all mice displaying clinical disease, indicating a systemic infection. Histopathological findings in these mice were characteristic of a fatal meningoencephalitis with focal necrosis of neurons, perivascular cuffing and infiltration of the meninges with neutrophils and mononuclear cells. However, no histopathological changes were noted in the lung, liver, heart, thymus, spleen, pancreas or kidney in BALB/c and CD-1 mice, despite widespread infection (94, 95). Outbred ICR mice had mononuclear cell infiltrates in the heart and liver and displayed medullary congestion and hemorrhaging in the kidneys, but there was no evidence of any renal or liver dysfunction as reflected by laboratory values (96).

Infant nude mice infected with hantavirus displayed identical clinical symptoms and virus localization, but a delayed onset of disease, suggesting that a mature cellular immune response may contribute to the pathology of disease (94). However, it is also clear that an anti-viral T cell response is important in control of infection. Intraperitoneal transfer of spleen cells from HTNV-immune mice protected infant mice inoculated i.c. 24 hours previously with a lethal dose of virus, demonstrating a critical role for T cells in the clearance of virus in these mice (94). Interestingly, in these studies of infant mice it was noted that survival time was inversely related to age, with death occurring significantly later in newborn mice (< 24 hours of age) than in mice inoculated at 5 or 7 days of age (94, 95). This observation was attributed to an incompletely developed immune system in the infant mice, and it was suggested that the cellular immune response may be involved in both immunopathology and protection against virus infection. The observation that immunocompetent CD-1 mice have earlier disease onset and more fulminant disease than their nude counterparts, despite identical virus titers in the brain, also supports a role for T cells in contributing to the pathogenesis of disease (95).
Immunocompetent mice greater than two weeks of age, in contrast to infant mice, do not exhibit any clinical symptoms and remain healthy following inoculation with HTNV. Viremia is transient in these mice (92), and the inability to detect viral antigens in any organ examined 4-6 weeks postinoculation indicates successful clearance of the virus (95). Adult nude mice, in contrast, become persistently infected following i.p. inoculation. In nude mice, viral antigen is detectable in lung, spleen and brain tissue but the mice show no signs of clinical illness. Transfer of spleen cells from immune mice to nude mice 1 day before inoculation prevented persistent infection, and depletion experiments demonstrated a critical role for CD8+ T cells in this protection (92).

E. PATHOGENESIS OF HFRS

The pathogenesis of hantavirus infection is not well understood. As mentioned previously, the lack of a suitable animal model has limited studies aimed at investigating the pathologic mechanisms underlying hantavirus disease. Many factors are likely to contribute to the syndromes that result from these infections. Some of these factors are discussed below.

1. Role of virus

The in vivo cellular targets in human hantavirus infections have not been completely characterized. Many cell types are permissive for hantavirus infection in vitro (97, 98), but in most of these the virus replicates to very low titers and requires a long replication period (11, 98, 99). Adaptation of HTNV to Vero E6 cells resulted in more efficient virus replication, and is thought to be the most suitable system for virus propagation and infectivity studies (11, 98).
In vitro studies have demonstrated that PUUV is able to infect human cell lines derived from lung (A-427, WI-38), kidney (A-704), liver (Hep G2) and pharynx (Detroit 562); as well as primary human glomerular cells, umbilical vein endothelial cells (HUVEC) and peripheral blood monocytes, thus demonstrating a wide host cell range (98-100). PUUV replication in most cell types was slow compared to replication in Vero E6 cells (peak infection at 2-3 weeks vs. 9 days) (98). Primary HUVEC cultures have been shown to be permissive for many different hantaviruses including HTNV, SEOV, PUUV, and Prospect Hill virus (PHV) by indirect immunofluorescence using patient sera (101). Hantavirus antigens were detected in approximately 20% of HUVECs on day 3 post inoculation, and in 100% of cells by day 10 post inoculation. A similar study demonstrated that human saphenous vein endothelial cells (HSVEC), as well as HUVEC and CV-1 cells, can be infected by HTNV (97). In that study, viral antigens and infectious virus were detected in all cell types by day 2-3 post inoculation. The amount of infectious virus produced by HSVEC and HUVEC cells peaked at day 3 post inoculation and declined gradually over the next 2-3 days, and a maximum of 4 logs of virus/ml was produced. No cytopathic effect was observed in any of the infected cultures during these studies (97, 98, 101).

Immunohistochemical studies on human tissues have demonstrated the presence of viral antigens in PBMC and kidneys during acute HFRS (102, 103), and lungs during acute HPS (75, 104). Yao and colleagues examined PBMC from 110 patients with acute HFRS and found viral antigens in 50.9% of the samples (102). Antigen were most often detected from days 4-7 after the onset of illness, and the percentage of positive cells varied from 2-20%. Infectious virus was isolated from the plasma and PBMC (B lymphocytes and monocytes) of these patients early in the course of illness (day 3-7 of illness), with the virus recovery rate from PBMC being twofold greater than from plasma. Other groups have also isolated virus from the peripheral blood monocytes of rats infected with HTNV (100), and monocytes have
been suggested to play an important role in the dissemination of virus in humans from the site of infection to the distant organs such as the kidney.

In rare, severe cases, viral antigen has also been detected in the cerebrospinal fluid (CSF), consistent with clinical findings of viral meningoencephalitis in these patients (102). HTNV-specific antibody and soluble HTNV antigen were detected in CSF, and intracellular HTNV antigens were detected in CSF monocytes in that report. These findings suggest that, in some circumstances, hantaviruses are able to traverse the blood-brain barrier and invade the central nervous system.

Immunohistochemical analysis of kidney biopsy samples from 23 patients with HFRS revealed the presence of viral envelope glycoproteins within the cytoplasm of renal tubular epithelial cells in greater than 95% of patients (103). Viral antigens were also detected in the capillary endothelium of interstitial areas and the glomerular capillary walls, although the staining was generally weaker than in the tubules. Viral glycoproteins were detected in renal tissues from days 5 to 30 after onset of fever. Pathologic findings in the kidneys included acute tubular necrosis and interstitial edema characterized by mononuclear cell infiltration and hemorrhage (103, 105). However, gross architectural abnormalities in the kidney were uncommon. Although there is some evidence of renal pathology in kidney biopsies, such as necrotic tubular epithelial cells that contain viral antigen (103), it is not clear whether necrosis is a direct result of the virus infection or an indirect result of the immune response to the virus.

It is generally accepted that hantaviruses are non-cytopathic in nature. Numerous studies have shown that hantaviruses, including HTNV, PUUV, SEOV, and PHV, do not have a direct cytopathic effect on cells infected in vitro (97, 98, 101, 106). However, one study reported that Vero E6 and renal carcinoma cells infected with HTNV or PHV showed signs of apoptotic cell death beginning at day 5 post infection (107). Vero E6 cells infected with
HTNV (MOI=0.5) had fragmented DNA as shown by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assays after 5 days of infection and fragmented DNA on agarose gels after 7-9 days of infection. The authors suggested that a decrease in the proto-oncogene protein Bcl-2, seen after 6 days of infection with HTNV, might be responsible for the apoptosis observed in this system (107). However, further support for the induction of apoptosis by hantavirus infection is lacking.

Autopsy samples from individuals with Sin Nombre virus infection showed evidence of predominant infection of alveolar microvascular endothelial cells as well as the lymphoid follicles of the spleen (75, 104). SNV antigen was also detected in the interstitial capillaries of the kidneys, but was absent from the tubular epithelial cells where viral antigen is predominantly located in HTNV infections (103). These studies demonstrated that infected cells are structurally intact, thus providing additional evidence that virus infection alone is insufficient to explain the capillary leakage that is characteristic of HFRS and HPS.

Although hantavirus infections appear to be non-cytopathic to the infected cells, the virus burden in an infected individual may impact the severity of disease. Our laboratory has quantitated SNV S segment RNA copies by RT-PCR in acute plasma samples from HPS patients and demonstrated a correlation between copies of viral RNA (vRNA) and severity of disease (108). In that study, 20 of 26 plasma samples obtained at the time of hospital admission were RT-PCR positive for SNV vRNA. When copies of SNV vRNA were compared to measures of disease severity, a significant positive correlation was found between vRNA copy number and peak hematocrit levels. In addition, a negative correlation was found between vRNA copy number and nadir platelet count. Finally the mean S segment RNA copy number was 1 log_{10} higher in the fatal cases than in survivors, although these values were not statistically significant. Taken together, these data suggested an association between higher virus burden and more severe illness during HPS caused by SNV.
2. Role of T lymphocytes

Recent studies of Hantaan and related virus infections in humans suggest a role for the cellular immune response in the pathology of HFRS and HPS. Studies of acute HFRS patients provide evidence of high levels of activation of the cellular immune response. Several groups have observed decreases in CD4:CD8 T cell ratios (75, 109, 110) as well as higher expression of T cell activation markers, including CD25, HLA DR and CD71, in serum during the febrile and oliguric phases of acute HFRS as compared to convalescence (111). Examination of biopsy samples revealed the presence of infiltrating lymphocytes (predominantly CD8+ T cells) in the kidneys of patients with acute PUUV infections (105, 112), and in the lungs of patients with fatal HPS caused by SNV (75). Although the function of these cells has not been formally studied, these infiltrating lymphocytes may be activated virus-specific T cells capable of recognizing virus-infected cells and secreting cytokines and chemokines. The presence of activated lymphocytes in the kidneys of infected individuals may result in local or systemic increases in production of these cytokines and other inflammatory mediators which may, in turn, contribute to the capillary leak syndrome and kidney damage characteristic of HFRS.

Additional evidence of T cell involvement in hantavirus pathology comes from a recent study by Mustonen and colleagues (113), which demonstrated a correlation between severe disease caused by PUUV infection and the HLA haplotype B8, DR3. This study included 74 patients with acute PUUV infections of varying severity. Of the seven of these patients who were in shock at the time of admission, all had the HLA B8, DR3 haplotype. Nine of the thirteen patients (69%) in the study required renal dialysis were HLA B8+, and eight (62%) were DR3+. Further, severe acute renal failure was observed in 15 (60%) of the HLA B8 positive patients compared to only 7 (15%) of HLA B8 negative patients. When
HLA haplotype was compared to other parameters of clinical disease, HLA B8, DR3 was found to correlate with several indicators of disease severity. A positive correlation was observed with maximum levels of serum creatinine and length of hospital stay, while an inverse correlation was seen with lowest blood pressure. This study showed that a striking association exists between a particular HLA type and more severe illness caused by PUUV infection. A similar relationship has been observed between HLA B35, DRB1402 and increased severity of SNV-induced HPS (114). Although these associations are likely to involve complex mechanisms, these findings suggest a role for T lymphocytes in exacerbating disease and support a potential role for the cellular immune response in the pathology of both HFRS and HPS.

Cytolytic T lymphocyte (CTL) lines have been isolated in our laboratory from patients with HPS caused by SNV infection (115). One CD8+ CTL line was isolated from the blood of a patient with acute HPS following in vitro culture of the PBMC with low levels of rIL-2. This CTL line recognized an epitope on the SNV N protein (amino acids 234-42) and was shown to secrete IFN-γ, but not IL-4, following stimulation with anti-CD3. Two additional SNV N-specific CTL lines that were isolated from a second HPS patient were also reported in that study (115). One of these was a CD4+ CTL line specific for SNV N_{372-80}, and the other was a CD8+ CTL line specific for SNV N_{131-39}. Interestingly, the latter CTL line was restricted by HLA B35, the HLA allele reported to be associated with more severe HPS caused by SNV infection (114). Additional B35-restricted CD8+ CTL lines have been isolated from HPS patient PBMC that recognize epitopes on SNV N or G2 proteins (unpublished data). These data demonstrated a heterogeneous T cell response to SNV N and/or G2 protein during acute HPS. These virus-specific CTLs may be involved in both the clearance of virus and immunopathology, although further studies are needed to define their role during HPS.
Prospective studies of dengue virus infection in Thai children have demonstrated that early immune activation contributes to the pathogenesis of dengue hemorrhagic fever (DHF), which is characterized by a capillary leak syndrome similar to that seen in hantavirus infection. Plasma levels of soluble tumor necrosis factor receptor type II (TNF-RII), soluble CD8 (sCD8), and soluble IL-2 receptor (IL-2R) were significantly elevated in children with DHF compared to the more mild dengue fever (DF) (116, 117). Levels of soluble TNF receptor (sTNF-R) also correlated with the degree of subsequent plasma leakage in children with DHF. In those studies, CD8+ T cell involvement in immunopathology is suggested by the increase in sCD8, sTNF-R and sIL-2R, which are shed from activated T cells (118-120).

Involvement of virus-specific CTL responses in both the clearance of virus and the induction of immunopathology has been observed in many virus systems including lymphocytic choriomeningitis virus (LCMV) (121, 122), herpes simplex virus (HSV) (123), rabies virus (124), and respiratory syncytial virus (RSV) (125).

In immunocompetent mice, i.e. inoculation with LCMV results in a fatal central nervous system (CNS) disease that is attributable to T cell mediumted destruction of infected cells (126). The fatal CNS disease is prevented if LCMV-specific CTL are transferred simultaneous with virus inoculation (13). In contrast, nude mice or immunodeficient mice inoculated in the same manner become persistently infected. Adoptive transfer of LCMV-immune splenocytes or LCMV-specific CTL clones into these persistently infected mice induces disease. These experiments clearly illustrate the dual role of virus-specific T cells in the protection from and immune pathology of LCMV.

In an experimental model of RSV infection, the transfer of RSV-specific CTL into infected mice facilitated the elimination of virus from the lungs of the mice, the rate of which was dependent on the number of transferred T cells (125). However, intravenous injection of large numbers of virus-specific CTL (8X10^6) was associated with augmented pulmonary
pathology that was fatal in 80% of mice. Histological examination of lung tissues in these mice revealed increased peribronchiolar infiltration of lymphocytes and monocytes compared to infected animals that received control influenza-specific T cells or no T cell transfer.

A complex role for virus-specific T cell responses has also been observed in patients infected with human immunodeficiency virus (HIV). In HIV infected humans, high CTL precursor (CTLp) frequencies and broad CTL responses have been associated with long-term non-progression in HIV, suggesting that CTL play an important role in controlling virus infection (127, 128). However, in a study of 37 patients with late-stage AIDS (≤50 CD4⁺ T cells/mm²), Giorgi and colleagues demonstrated that the level of CD8⁺ and CD4⁺ T cell activation, but not virus burden, was associated with a shorter mean survival time (129). In that study, higher proportions of activated CD4⁺ and CD8⁺ T cells expressing the activation marker CD38 were found in patients who survived <6 months after CD4⁺ T cells dropped below 50/mm², compared to those who survived >18 months. In addition, CD38 expression was higher on a per cell basis in patients with shorter survival times. In another study, elevated levels of CD38⁺ CD8⁺ T cells were correlated with increased virus burden in HIV-seropositive men (130). These observations suggest that virus-specific T cells may contribute to progression of HIV disease, although the mechanism by which this might occur remains unknown.

3. Role of cytokines

There are several reports of local and systemic increases in cytokine production during acute hantavirus illness. A study of sera from 110 HFRS patients collected during the Korean conflict in the 1950s demonstrated elevations in levels of IL-1β, IFN-γ and IFN-α in HFRS patients compared with normal controls (131, 132). Another study of 15 patients with acute
PUUV infections revealed increases in plasma levels of IL-6 and TNF-α in all patients, and increases in IL-10 levels in 13 of 15 patients during the acute phase of illness compared to convalescence (133). Levels of TNF-α remained elevated at day 8 after onset of fever in 11 patients in this study, while levels of both IL-6 and IL-10 had significantly decreased in all patients by this time. Increases in IFN-γ and IL-8 were also observed in a minority of patients in that study. Interestingly, levels of TNF-α were found to inversely correlate with the ratios of the mean blood pressure recorded at admission and at 3 months, suggesting a possible contributing role for TNF-α in disease severity.

Indirect immunofluorescence studies of renal biopsies from patients infected with PUUV showed increased levels of TNF-α and TGF-β in the kidneys of patients with acute NE, which co-localized with inflammatory cell infiltrates, potentially implicating the infiltrating cells as the primary source of these cytokines (112). That study also demonstrated increased expression of ICAM-1, VCAM-1 and PECAM in peritubular regions, which colocalized with both TNF-α expression and cellular infiltrates. Expression of adhesion molecules is also increased in the kidney during other immunolopathological renal diseases, such as murine lupus nephritis (134). Increased expression of adhesion molecules is thought to contribute to the “stickiness” of the activated endothelium in this model, thus contributing to leukocyte infiltration.

Preliminary in vitro studies of hantavirus infection have demonstrated that virus infection alone may induce the expression of cytokines and chemokines by the infected cell (135). In one study, the mRNA expression of the chemokines MCP-1, MIP-1α, RANTES and IL-8 was induced in HUVEC and primary human monocytes following infection with the pathogenic ANDV and SNV, but not with the non-pathogenic PHV (135). In this system, expression of mRNA for TNF-α and IFN-γ was not induced by virus infection. Another
study demonstrated the induction of RANTES in a human epithelial kidney cell line (293HEK) and GM-CSF in a fetal lung fibroblast cell line (MRC-5) following infection with HTNV (136). HTNV infection of primary human monocytes in that study also induced RANTES secretion and upregulated mRNA expression of the RANTES receptors CCR1 and CCR5. SNV or ANDV infection, in contrast, induced production of TNF-α from a human monocytic cell line (THP-1) and HUVECs, and the production of MCP-1 in 293HEK cells. These studies are preliminary but they clearly demonstrate the potential of various permissive cell types to produce cytokines and chemokines as the result of hantavirus infection. The production of chemokines and cytokines from infected cells could facilitate the recruitment of lymphocytes and monocytes to the sites of infection, and contribute to the amplification of the immune response.

Immunohistochemical analysis of autopsy samples from 6 fatal cases of HPS revealed increased numbers of cells staining for inflammatory cytokines, including IL-1α, IL-1β, IL-6 and TNF-α (137). These cytokines were detected in cells in both the alveolar walls as well as in the alveolar spaces. Lymphokines such as IFN-γ, IL-2, IL-4 and TNF-β were also detected in small cells in the alveolar walls. Other tissues also contained cytokine-producing cells, including the kidney, liver and spleen.

Although cytokines are a crucial component of an effective anti-viral immune response, the overproduction of cytokines can have detrimental effects on the host and, in some cases, exacerbate illness. The role of cytokines, including TNF-α, IFN-γ, IL-1 and IL-2, in mediating immunopathology is well documented.

TNF-α is a pleiotropic cytokine, produced primarily by macrophages, that mediates the necrosis of certain tumors in mice following treatment with bacterial LPS. Clinical trials carried out to assess the potential therapeutic anti-tumor properties of TNF-α in advanced
cancer patients revealed that intravenous infusion of TNF-α resulted in the development of fever, myalgia, thrombocytopenia and hypotension (138). High doses of TNF-α (205 μg/m^2) administered alone or in combination with IFN-γ caused severe, sometimes life-threatening hypotension resulting from peripheral edema which is thought to be due to vascular leak (138, 139). Pulmonary toxicity was also reported in that study, as reflected by dyspnea, tachypnea and severe hypoxemia. TNF-α treatment also caused symptoms such as fever, fatigue, headache and myalgias in the majority of subjects. Many of these constitutional symptoms and clinical complications are similar to the clinical findings during HFRS and HPS.

TNF-α has also been identified as the primary effector molecule in the pathology of septic shock. Plasma levels of TNF-α were elevated in the plasma of septic shock patients and in volunteers injected intravenously with bacterial endotoxin (140). In rats and sheep, administration of recombinant TNF-α recapitulates the symptoms of bacterial septicemia, including severe hypotension and vascular leak (141, 142). These symptoms are also features of severe, acute hantaviral diseases. Infusion of monoclonal antibodies to TNF-α prior to treatment with endotoxin completely protects animals from these effects, implicating TNF-α as the primary effector molecule involved in mediating shock in these models (141-143).

Elevated levels of TNF-α have also been reported in the serum of patients infected with hemorrhagic fever viruses including Dengue virus and Ebola virus (144). Both viruses are characterized by a capillary leak syndrome, and dengue hemorrhagic fever has many similar clinical features in common with HFRS and HPS. In Dengue virus infection, TNF-α was found to be elevated in the serum of patients with the severe disease Dengue hemorrhagic fever (DHF) as well as the milder disease Dengue fever (DF) compared to controls (145, 146). TNF-α, as well as IFN-γ and IL-2, were found to be significantly elevated in the serum of patients with fatal Ebola virus infections compared to patients with nonfatal infections and
normal controls (144). In an *in vitro* study of filovirus infection, supernatants from Marburg virus-infected monocytes induced leakiness in an endothelial cell (EC) monolayer (147). This effect was inhibited when an anti-TNF-α monoclonal antibody was added, demonstrating that TNF-α was responsible for the leakiness of the ECs.

TNF-α is also known to have additional effects on ECs, which may provide insight into the mechanisms that underlie its pathologic effects. Since capillary leak is a hallmark of hantavirus disease, and endothelial cells appear to be a primary target of hantavirus infection, the effects of TNF-α on these cells are important to consider. TNF-α has been shown to cause the upregulation of MHC class I molecules as well as adhesion molecules such as ICAM-1 and ELAM-1 on the surface of ECs (148). The upregulation of these molecules contributes to an increased adhesiveness of lymphocytes and other cell types to ECs (149).

TNF-α also induces the production of cytokines by ECs, including IL-1, IL-6, MCP-1 and IL-8, that may contribute to vascular permeability (IL-1) and recruit additional immune cells to the endothelium (MCP-1, IL-8), thus amplifying the inflammatory response.

TNF-α has also been shown to induce morphological changes in endothelial cells, which could account for increases in EC permeability (148). Recombinant TNF-α (rTNF-α) treatment has been shown to cause a reversible redistribution of plakoglobin, a protein found in the intra-endothelial cell junctions (147). This redistribution is indicative of the formation of intra-endothelial cell gaps and correlates with increases in permeability. Changes in structural proteins such as plakoglobin are likely to result in decreased contact between neighboring EC, as well as in decreased adherence to the cytoskeleton.

IFN-γ has many immunoregulatory effects including activation of macrophages to enhance phagocytosis and anti-tumor activity, activation of cytolytic T cells and NK cells, and induction of class I and II MHC molecules on various cell types. IFN-γ has also been shown
to synergize with TNF-α to promote capillary permeability (138). IFN-γ also appears to synergize with TNF-α in the activation of endothelial cells. In vitro studies have shown that treatment of endothelial cell monolayers with a combination of IFN-γ and TNF-α causes reversible morphological changes in these cells such that they become plump and retract from one another, forming dendrite-like processes and intercellular gaps (148). These morphological changes may contribute to increased permeability. Like TNF-α, IFN-γ also stimulates the upregulation of MHC class I molecules on endothelial cells which likely results in enhanced interactions between T cells and the endothelium.

Serum IFN-γ levels were shown to be significantly elevated in patients with lethal Ebola virus infections relative to levels in patients who survived infection (144). Similarly, in studies of acute dengue virus illness, IFN-γ was shown to be elevated in the sera of patients with dengue hemorrhagic fever and dengue fever compared to normal controls (117, 150).

Other cytokines such as IL-1 and IL-6 have been noted to be elevated during acute HFRS. These inflammatory cytokines can activate T and B cells, and can synergize with other cytokines and each other to further elevate the immune response. IL-1 and IL-6 synergize with each other to cause increased production of IL-2 and increased expression of IL-2R on T cells, which could cause amplification of T cell activation and proliferation (151). IL-1β is also known to contribute to the activation of ECs and, either alone or in combination with TNF-α, enhance interactions of T and B cells with endothelial cells (149, 152). IL-1-mediated increases in EC adhesiveness for lymphocytes may result from the marked upregulation of ICAM-1 on EC following IL-1 treatment (153). Not surprisingly, IL-1β also synergizes with TNF-α to produce hemodynamic changes associated with endotoxic shock in rabbits (154) and has been shown to be elevated in patients with septic shock (140).
IL-10, which has been noted to be elevated during HFRS (133), was also shown to be elevated in children with DHF, compared to those with uncomplicated DF (155). In that study, levels of IL-10 were significantly correlated with the degree of plasma leakage as measured by pleural effusion index. IL-10 is a potent activator of B lymphocyte differentiation, and an inhibitor of T cell proliferation (156). IL-10 is also a potent inhibitor of IL-12 production and can thus promote the development of a Th2-type T cell response (157). During HFRS, one could speculate that IL-10 may act as a regulatory cytokine that is produced to counteract the effects of elevated TNF-α.

4. Role of HLA haplotype

The association of a particular HLA haplotype with severity of disease suggests a potential role for the host cellular immune response in disease pathogenesis. Several possible explanations for a more severe disease course related to HLA haplotype may be envisioned. It is possible that individuals with certain HLA alleles have a more vigorous immune response to the virus, possibly due to the number of potentially antigenic peptides within the viral proteins that are able to bind to a specific HLA molecule. An extremely intense immune response could lead to an overproduction of cytokines and inflammatory mediators, as well as continued activation of lymphocytes, that could contribute to immunopathology. Alternatively, a dysfunctional immune response to the virus could delay the clearance of the virus and allow enhanced virus replication.

As discussed earlier, a recent study conducted by Mustonen and colleagues (113) demonstrated an association between the HLA haplotype B8, DR3 and more severe illness following PUUV infection. The same group subsequently noted a significant correlation between this haplotype and the ability to detect PUUV RNA in the blood and urine of patients
by PCR (158). In this smaller study, PCR positivity was correlated with several measures of disease severity, including maximum levels of serum creatinine, maximum blood leukocyte counts, the need for dialysis and the length of hospitalization. The increased ability to detect viral RNA in patients expressing the HLA B8, DR3 alleles suggests that these patients may have a higher virus burden, although levels of viremia were not quantitated in these individuals.

The mechanism underlying the association between HLA and disease severity is not defined, and is likely to be complex and multifactorial. A polymorphism in the TNF-α promoter region that confers increased TNF-α production (TNF2 allele) was found more commonly among HFRS patients (42%) than among normal controls (15%) (159). Further, among HFRS patients the TNF2 allele was found in 69% of patients requiring dialysis as compared to only 15% among patients who did not require dialysis. The TNF2 allele was also found in all patients who experienced shock. The TNF2 allele differs from the more common TNF1 allele by a single g→a base change at position -308 relative to the transcriptional start site of the TNF-α gene. This TNF-α polymorphism has been shown to be strongly associated with the HLA haplotype A1,B8,DR3 (160), the same haplotype associated with severe HFRS following PUUV infection. The data from this study do not include measurements of serum TNF-α levels in patient sera, and the observed differences in the clinical course of HFRS cannot necessarily be attributed to the TNF-α polymorphism. In the study by Kanerva and colleagues, the presence of the TNF2 allele was significantly correlated with certain measures of disease severity, including maximum serum creatinine levels and lowest platelet count. However, since the TNF2 allele is in linkage disequilibrium with the HLA haplotype B8, DR3, it was not possible to determine which factor was most strongly associated with disease severity. More recent analysis of a larger patient population (n=116)
by the same group revealed that the HLA B8, DR3 haplotype is more strongly associated with severe HFRS caused by PUUV than the TNF2 allele (161). In that study, analysis of various clinical parameters of disease severity showed a significant correlation between HLA B8, DR3 and maximum creatinine and maximum leukocyte count during the course of treatment. In contrast, no correlations were observed between clinical measures of disease severity and the possession of the TNF2 allele, suggesting that the HLA haplotype B8, DR3 is a more important factor in the severity of HFRS caused by PUUV infection.

The extended haplotype A1, B8, DR3 has also been associated with autoimmune diseases including systemic lupus erythematosus (SLE), celiac disease, multiple sclerosis, thyroiditis, Addison’s disease, type-1 diabetes mellitus and chronic active hepatitis. In most of these autoimmune diseases, inappropriate activation of T lymphocytes is thought to contribute to disease (162, 163). In individuals with active SLE, increased percentages of activated CD4+ T cells are found in the circulation, but these cells show depressed proliferative responses to mitogens in vitro. In contrast, CD8+ T cells from SLE patients did not express activation markers and were found to respond normally to mitogen stimulation, although overall numbers of circulating CD8+ T cells were significantly lower than in normal controls or individuals with inactive SLE (162). Activated T lymphocytes are also thought to play a key role in the pathology of multiple sclerosis (MS), since CD25+ T lymphocytes were found in the cerebrospinal fluid of patients with active MS, but were absent in patients with chronic MS or other neurological diseases (163). Although it is clear that T cell responses were altered in patients with these autoimmune conditions, the specific mechanisms responsible for the HLA B8, DR3 associated predisposition to autoimmunity have not been defined.

Individuals who carry this haplotype have also been shown to have abnormal T cell responses in vitro. Studies of healthy young adults carrying the A1, B8, DR3 haplotype
demonstrated that PBMC from these individuals had impaired proliferative responses to stimulation with mitogens as compared to controls (164, 165). Mitogen stimulated PBMC from individuals with this haplotype were also shown to secrete less IL-2 than controls, and this was thought to account for the limited proliferation. It has also been noted that the secretion of other cytokines, including IL-1, IL-5 and IFN-γ, is significantly impaired in these individuals. However, no differences were noted in production of IL-4, IL-6 or IL-10 between HLA B8/DR3+ and HLA B8/DR3- individuals (166, 167). Another study has shown that expression of the early activation marker CD69 following PHA stimulation is also defective in lymphocytes from HLA B8, DR3+ individuals.

In mice, it is clear that differences in MHC haplotype impact the level of immune responsiveness to hantaviruses. A recent study examined humoral and T cell responses in mice following immunization with a recombinant PUUV N protein (168). This study showed that H-2k mice had the strongest antibody responses following immunization, followed by H-2d and, lastly, H-2b mice. The same hierarchy was observed for T cell proliferative responses following in vitro restimulation with pools of overlapping PUUV N peptides. This study illustrated that the immune response to hantavirus infection is impacted by the MHC haplotype of the infected mouse, probably due to differences in the ability of particular HLA alleles to present determinants from the hantavirus proteins.

F. HANTAVIRUS VACCINES

Currently there is no effective vaccine against hantavirus infection that is widely administered. Several vaccines utilizing inactivated HTNV and SEOV virus grown in cell culture or mouse brain have been tested in humans in Asia (169-171). These vaccines have been shown to be safe and to elicit neutralizing IgG responses. However, antibody responses
in vaccine recipients tended to be low and transient with <50% of vaccinees retaining measurable antibody responses one year after the initial vaccination in one study (169). Other strategies have employed vaccinia virus or baculovirus systems to express recombinant hantavirus antigens (172-174). Inoculation with a recombinant vaccinia virus expressing the HTNV M genome segment was shown to elicit neutralizing antibody responses in mice (173, 174) and to protect hamsters against subsequent infection with native HTNV (173). Passive transfer of monoclonal antibodies to the glycoproteins also protected hamsters from challenge with HTNV, suggesting that neutralizing antibody responses may be sufficient to protect against infection (173). Recombinant vaccinia viruses expressing the HTNV S genome segment appeared to be less effective in both eliciting antibody responses in mice and in protecting hamsters against HTNV infection. However, immunization with a recombinant baculovirus expressing the HTNV S segment was shown to be effective in eliciting antibody responses as well as in protecting hamsters against subsequent infection (173). In a recent clinical trial in humans, subcutaneous (s.c.) inoculation with a vaccinia virus expressing the HTNV M segment elicited neutralizing antibody (nAb) responses in 72% of vaccinia virus-naive volunteers, and 26% of vaccinia virus-immune volunteers (175).

The cellular immune response has also been shown to play a role in protection against hantavirus infection. In one study, passive transfer of spleen cells from mice immunized with baculoviruses expressing HTNV S (rNP) or M (rEnv) segments to suckling mice conferred partial protection and prolonged survival time after lethal HTNV challenge. This protection was also noted when monoclonal antibody or T cell-enriched spleen cell fractions from rNP immunized mice were transferred, indicating a role for both T lymphocytes and antibody responses in the protective effect. Phase I and Phase II clinical trials in humans also resulted in the development of CD4+ T cell responses following s.c. inoculation with a vaccinia virus expressing the HTNV M segment (175).
Recently, portions of the nucleocapsid protein from PUUV were expressed in hepatitis B virus core-derived chimeric proteins, and immunization with these particles was shown to protect bank voles against subsequent infection with a different strain of PUUV (176). Naked DNA vaccines have also been recently developed. DNA constructs expressing the M or S genome segments of SEOV were tested for immunogenicity in BALB/c mice and Syrian hamsters. Inoculation with DNA vaccines expressing either the M or S segment resulted in the production of virus-specific antibody in mice, with gene gun administration proving to be the most effective route of inoculation (177). When Syrian hamsters were vaccinated with these constructs and subsequently challenged with SEOV, only the construct expressing the M segment effectively conferred protection against virus challenge, although both constructs elicited antibody responses in the animals.

Most of the immunization strategies developed thus far focus on the development of a neutralizing antibody response, and this response has been demonstrated to be effective in some systems. However, the studies of humans inoculated with inactivated hantavirus vaccines demonstrate that the antibody response to these virus formulations may be low and transient. Additional studies in human subjects will be required to determine whether other vaccine strategies will result in the development of more long-lasting anti-viral antibody responses in humans. Ultimately, the development of a vaccine capable of inducing both T cell responses as well as neutralizing antibody responses may be required to insure long-lived protection against hantavirus infection.

G. THESIS OBJECTIVES

HFRS and HPS have been suggested to be caused in part by immunopathologic mechanisms, including the actions of CD8+ and CD4+ T lymphocytes. We hypothesize that a
combination of very intense virus-specific T cell responses and high virus titers in infected individuals leads to the development of an immunopathologic state that may contribute to hantavirus disease. T lymphocytes may play a dual role during hantavirus infection, as they do in many other viral infections. It is likely that an effective T cell response is critical for the clearance of virus. However, it is also possible that an extremely robust T cell response may lead to a relative “over-activation” of the immune system, which could contribute to immunopathology. Quantitative analyses of virus-specific T cell responses during acute hantavirus infections have not been performed and will be critical in determining whether a correlation exists between levels of T cell activation and disease severity.

The overall goal of the studies discussed here is to gain an understanding of the scope and characteristics of the memory T lymphocyte response to Old World hantavirus infections by identifying dominant viral proteins and T cell epitopes that are targeted by the CTL response in immune individuals. Prior to the present studies, no formal analyses of T lymphocyte responses to these viruses had been performed. The studies described here not only provide the first qualitative and quantitative information regarding virus-specific human T cell responses to HTNV and PUUV, they also provide the necessary framework to conduct quantitative studies of specific T cell responses during acute illness, using techniques such as MHC-peptide tetramer analysis and intracellular IFN-γ staining. Such quantitative analyses of the magnitude and dynamics of the virus-specific T cells responses during acute infection will be critical to the understanding of the role that T cells play in the clearance of virus and in the pathology of disease.

Further, the identification of viral proteins and T cell epitopes that are targeted by the human immune system may impact the design of effective vaccines that can elicit both T
lymphocyte responses and antibody responses. The specific objectives of this thesis are as follows:

1. Characterization of the memory T cell responses in individuals with sub-clinical, laboratory-acquired infections with HTNV.


3. Design and optimization of a quantitative, competitive RT-PCR strategy to measure viral RNA levels in patient plasma during acute PUUV infection.
CHAPTER II
MATERIALS AND METHODS

A. PBMC Donors.

*Hantaan virus study:* Donors were American scientists who became infected with the HTNV 10-15 years prior to the study. Infections were subclinical and were confirmed by detection of HTNV-specific immunoglobulin in the serum. The HLA types of these donors are as follows: Donor A: A1, A2, B35, B51, Cw4, Cw7, DR2, DR3, DQ2, DQ6 and DRw52. Donor B: A2, A11, B39, B60, Cw3, Cw12, DR4, DR9, DQ3 and DRw53. Donor C: A1, A11, B8, B51, Cw7, Cw15, DR7, DR11, DQ3, DRw52 and DRw53.

PBMC were purified by Ficoll-Hypaque density gradient centrifugation. Buffy coats were recovered and mononuclear cells were resuspended at 5-8×10^6/ml in RPMI 1640/20% FBS/10% dimethyl sulfoxide (DMSO) and cryopreserved until needed.

*Puumala virus study:* Donors were Finnish individuals who had clinical HFRS caused by PUUV infection between the years 1984 and 1995. Diagnosis of PUUV infection was confirmed by serological detection of PUUV-specific antibodies using a commercially available ELISA kit. PBMC were purified using Vacutainer CPT tubes (Becton Dickinson). Cells were resuspended at 1-2×10^7/ml in RPMI 1640 supplemented with 20% FBS (Sigma, St. Louis, MO), 2mL L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 10% DMSO and cryopreserved until use. The age, year of infection and Class I HLA haplotypes of the donors are shown in Table IV-1.
B. B lymphoblastoid cell lines (BLCLs).

PBMC (1-4X10⁶) were cultured in RPMI 1640, supplemented with penicillin, streptomycin, L-glutamine, HEPES and 20% FBS in the presence of 1:3 dilution of Epstein-Barr virus from B95-8 cells (ATCC) in 24-well flat-bottom plates (Costar), as previously described (178). Cyclosporin A was added at 1µg/ml to inhibit proliferation of T lymphocytes. Once transformed, BLCLs were maintained in RPMI 1640/20% FBS and passaged three times/week.

C. Recombinant vaccinia viruses expressing hantavirus proteins.

Recombinant vaccinia viruses expressing HTNV genes were constructed as described (173) and were kindly provided by Dr. Connie S. Schmaljohn. The recombinant vaccinia viruses used in this study were derived from the WR strain of vaccinia and express either individual HTNV genes encoding G1, G2 or nucleocapsid (denoted vac-G1, vac-G2 and vac-N, respectively), or all three genes simultaneously (denoted vac-G1+G2+N).

Recombinant vaccinia viruses expressing PUUV genes were constructed as described (Masanori Terajima, manuscript in preparation), and express whole or partial PUUV genes encoding N, G1, G2Bs (amino acids 1-256) or G2/Sm (amino acids 227-490).

D. Synthetic peptides.

Seventy (70) HTNV nucleocapsid peptides were synthesized that spanned the entire published sequence of the HTNV nucleocapsid protein (15)(Accession#M14626). Peptides were 15 amino acids in length and overlap by 9 amino acids. Seventy (70) PUUV nucleocapsid peptides were synthesized as above based on the published sequence of PUUV strain K27 (179) (Accession#L08804). N protein peptides from other hantavirus strains were synthesized
based on published sequences and included SEOV (18) (accession#M34881), SNV (180) (accession#L37904), PHV (181) (accession#M34011), ANDV (8) (accession#AF004660), PUUV strain Hallnas B1 (182) (accession#M32750), NYV (183) (accession#U47135), and DOBV (184) (accession#L41916) and SNV strain CC107 (185) (accession#L33683). Peptides were synthesized at the Protein Chemistry Core Facility at the University of Massachusetts Medical School using an automated Rainin Symphony peptide synthesizer.

E. In vitro stimulation of PBMC.

Hantaan virus study: 2-5X10^6/ PBMC were plated in 1 ml AIM/V/ 10% human AB serum in a single well of a 24-well plate (Costar). A gamma-irradiated, inactive HTNV preparation was added at a 1:160 final dilution. HTNV was grown in Vero E6 cells (ATCC) and purified as described (11). The purified virus was then γ-irradiated (8x10^6 Rads) to inactivate the virus. Inactivation of the virus was confirmed by plaque assay. Recombinant human IL-2 (20U/ml) was added on day 4 or 5 in 1 ml of fresh AIM/V/10% human AB serum and the medium was replenished every third day thereafter. Bulk culture ^51^Cr-release assays were performed between days 7 and 14 of culture. Donor A PBMC was restimulated on day 7 with inactivated HTNV (1:160) and 2-3X10^6 γ-irradiated autologous PBMC in order to reduce non-specific background lysis. rIL-2 was added to these cultures on day 11 and cultures tested on day 14.

Puumala virus study: 5-7X10^6 PBMC were resusupended in 1 ml of AIM-V medium supplemented with 10% human AB serum. The PBMC were stimulated with 20 μl of purified PUUV (strain K27, kindly provided by Connie S. Schmaljohn), grown in Vero E6 cells and purified as described (11). Recombinant human IL-2 (5 U/ml) was added on day 5-6 of culture in fresh AIM-V/10% human AB serum and the medium was replenished every third
day thereafter. Bulk $^{51}$Cr-release assays were performed on day 7-8 of culture. In some cases, cultures were restimulated once with $2 \times 10^6$ γ-irradiated autologous PBMC and 20 μl of purified PUUV in fresh AIM-V/10% HuAB serum supplemented with 10 U/ml rIL-2. $^{51}$Cr-release assays were performed on restimulated cultures 7-8 days post-restimulation (Day 14-15 of culture). Cultures were maintained and all experiments were performed in a biosafety level 3 (BSL3) laboratory according to standard BSL3 guidelines.

F. CTL lines.

HTNV-specific or PUUV-specific CTL lines were established by limiting dilution plating as described (186). *In vitro* stimulated PBMC were plated at 1, 3, 10 or 30 cells/well in a 96-well, round-bottom plate (Costar) in 0.2 ml AIM/V/10%FBS and stimulated using anti-CD3 antibody (12F6) and gamma-irradiated allogeneic PBMC as feeder cells. Medium was replenished every three days and wells were restimulated with anti-CD3 and γ-irradiated feeder cells every 14 days. Individual wells were tested for recognition of target cells expressing HTNV or PUUV proteins in a $^{51}$Cr-release assay and positive wells were expanded and restimulated as described above. Surface expression of CD4 and CD8 were determined by flow cytometry using fluorescein isothiocyanate-conjugated antibodies (Becton Dickinson). HLA restriction of individual CTL lines was determined using partially HLA-matched BLCL targets infected with a vaccinia virus recombinant expressing HTNV or PUUV N. Fine specificity of CTL lines was determined using overlapping synthetic peptides that span the HTNV or PUUV N protein and subsequent N- and C-terminal peptide truncations.
G. Preparation of target cells.

*Virus-infected targets:* Autologous or allogeneic (for MHC restriction analysis) B lymphoblastoid cell lines (BLCL) were infected with recombinant vaccinia viruses at an MOI=15 for 60 mins at 37°C. The cells were then diluted in 1 ml of RPMI 1640/10% FBS for an additional 12-16 hours. Target cells were then labeled with 0.25 mCi of $^{51}$Cr for 60 mins at 37°C. Following labeling, the cells were washed 3 times and resuspended at 1-2X10^6 cells/ml in RPMI/10% FBS.

*Peptide-pulsed targets:* Uninfected BLCL were $^{51}$Cr labeled as described above. Labeled cells were incubated with the indicated concentrations of peptide in 96-well round-bottom plates at 1-2X10^3/well for 30 mins at 37°C before addition of effector cells. The peptides remained in the wells for the duration of the assay.

H. $^{51}$Cr-release cytotoxicity assay.

*In vitro* stimulated effector cells were added to 2X10^3 $^{51}$Cr-labeled target cells at the indicated E:T ratios. For CTL assays using synthetic peptides, T cell lines or clones were added to 1-2X10^3 $^{51}$Cr-labeled, peptide-pulsed targets at an E:T ratio of 10:1 (unless otherwise specified). Spontaneous release was measured in wells containing target cells and medium, and maximum release was measure in wells containing target cells and 5% Renex. Plates were incubated for 4.5 hours at 37°C, supernatants harvested (Skatron Instruments, Sterling, VA) and specific lysis calculated as ($(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release}) \times 100$). All targets were tested in duplicate or triplicate wells. All experiments were performed at least twice. Negative controls included target cells infected with wild type vaccinia virus or unpulsed target cells. Spontaneous lysis was <25% in all assays.
I. Proliferation Assays.

PBMC. Convalescent PBMC from PUUV-immune individuals were stimulated in vitro with γ-irradiated PUUV at the indicated dilutions at 37°C. PBMC were seeded at 1.5X10^5/well in 96-well round bottom plates in replicates of 5. Six days post-stimulation, 50 μl of supernatant was collected from each well for analysis of cytokine secretion. The wells were then pulsed with [3H]-thymidine (1.25 μCi/well) and incubated for an additional 16 hours. The highest and lowest counts per min (CPM) values were discarded and the remaining 3 values were averaged. Stimulation index was calculated as CPM (virus stimulation)/CPM (medium control).

J. Cytokine analysis by ELISA.

Supernatants were collected from cultures of PBMC stimulated with γ-irradiated PUUV (Day 6) or T cell clones stimulated with peptide-pulsed autologous BLCL (48 hours), as described above. Quantitation of cytokines in supernatants was performed using commercial kits (Endogen) according to the manufacturer’s instructions.

K. ELISPOT assay for single cell IFN-γ secretion.

ELISPOT assays were performed as described (187). Briefly, 96-well filtration plates (MAIP S 45; Millipore, Bedford, MA) were coated with 15 μg/ml mouse anti-human IFN-γ monoclonal antibody (mAb) (clone NIB42, Pharmingen, San Diego, CA). PBMC were incubated for 24 hours in RPMI/10% FBS with no stimulation and added at 2X10^5 cells/well in RPMI 1640/10% FBS. Peptides were added at 25 μg/ml and plates were incubated for 18-20 hours at 37°C. Following extensive washing (6X with PBS/0.5% Tween), biotinylated
mouse anti-human IFN-γ mAb (clone 4S.B3, Pharmingen) was added to each well and plates were incubated for 2 hours at room temperature. Plates were washed extensively (8X with PBS/0.5% Tween) and then a 1:400 dilution of streptavidin alkaline phosphatase was added and plates were incubated at room temperature for 45 mins. Substrate (3-amino-9-ethylcarbazole/0.15%H₂O₂; 200 µl) was added to each well and plates were incubated for 10 mins at room temperature. Precursor frequency was calculated as #visible spots/# total cells per well. Experiments were performed in triplicate.

L. TCR Vβ expression by PCR.

Total cellular RNA was isolated using Ultraspec total RNA isolation reagent (Biotecx Laboratories, Inc, Houston, TX) according to manufacturer’s instructions. cDNA was synthesized using random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The cDNA was then aliquoted into 26 tubes, each containing a single T cell receptor (TCR) Vβ-specific primer and a β constant region-specific primer. Each tube also contained primers specific for β-actin as an internal control. PCR was performed for 25 cycles as follows: 95°C for 1 min., 55°C for 1 min., 72°C for 1 min (1.5 min. denaturing step used for the first cycle). PCR reaction products were resolved on a 5% polyacrylamide gel and visualized by ethidium bromide staining.

M. Plasmid construction for quantitative RT-PCR.

Construction of competitor plasmid. To construct the competitor S segment RNA transcription vector, we started with the pFIR2+118 plasmid (108), which contains the Sin Nombre virus S segment (nt 42-309) plus an additional 118 base pairs (bp) from the ØX174/HaeIII molecular weight marker. In order to use this competitor plasmid for PUUV-
specific RT-PCR, we engineered PUUV-specific primer binding sites at the ends of the SNV S segment as follows. The pFIR2+118 plasmid was first digested with HaeIII for linearization and the SNV S segment (including the 118-bp insert) was amplified by PCR using hybrid primers containing both SNV- and PUUV-specific regions. The PUUV-specific portions of the primers was chosen from a conserved region of the PUUV S segment (Sotkamo strain; accession #X61035) and span nucleotides 63-86 (forward primer) and 286-309 (reverse primer) of the S segment (underlined regions below). The SNV specific portions of the primers span nucleotides 87-97 (forward) and 275-286 (reverse) of the SNV S segment (accession #L25784). The primer sequences were as follows: forward primer (PUUSNVF): 5’-CCA AGA GGA TAT AAC CCG CCA TGA ACA ACA ACT CG-3’; reverse primer (PUUSNVR): 5’-GTG GTC ATC AGGTTC AAT CCC AGT TGG ATC AAC AG-3’. The resulting 388-bp PCR product was cloned into the pCR2.1 vector using the TA Cloning kit (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. A plasmid that contained the PCR product in the same orientation as the lacZ gene, designated FIR2+118-8, was confirmed by restriction enzyme digestion and selected to produce negative-stranded RNA by in vitro transcription.

Construction of positive control plasmid. To generate positive control RNA for the RT-PCR assay we used a plasmid, pSP72-1, which contains the cDNA for the PUUV S segment (strain K27, accession #L08804) inserted into the EcoRI site within the multiple cloning site of plasmid pSP72. Since we were unable to efficiently transcribe RNA from the full-length linearized template, we truncated the PUUV S segment in pSP72-1 via restriction enzyme digestion with AccI. This digestion removed the 3’ end of the S segment cDNA leaving nucleotides 1-360. The resulting plasmid was then religated and was designated pSP72-1tr.
N. *In vitro* transcription.

The FIR2+118-8 (competitor) and pSP72-1tr (positive control) plasmids were digested with *BamH*I and *EcoRV* enzymes respectively. The linearized templates were used for *in vitro* transcription, which was carried out using the Ribomax Large Scale RNA Production System – T7 or – SP6 (Promega) according to the manufacturer's instructions. The competitor RNA was transcribed from the T7 promoter and the positive control from the SP6 promoter. The *in vitro* transcribed RNA was treated with RQ1-RNase free DNase and purified using Ultraspec total RNA isolation reagent (Biotex Laboratories, Houston, TX). Spectrophotometric analysis (OD 260/280) was used to assess the concentration and purity of the RNA. The RNA was aliquoted and stored at −70°C until use.

![Diagram of expression plasmids](image_url)

**Figure II-1:** Expression plasmids used for *in vitro* RNA transcription. PSP72-1tr (positive control plasmid) and FIR2+118-8 (competitor plasmid).
O. RNA Extraction

RNA was extracted from 140 μl of human plasma using the QIAamp Viral RNA kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The RNA was eluted in 50 μl of diethylpyrocarbonate-treated water and stored at −70°C until use.

P. RT-PCR.

The PCR primer sequences were designed to bind to relatively conserved regions of the PUUV S segment based on sequence alignments of the S segments from multiple PUUV strains. The PCR primers were identical to the PUUV-specific portion of the hybrid primers described in section N of the Methods. The primer sequences were as follows: upstream primer (PUU6386): 5'-CCA AGA GGA TAT AAC CCG CCA TGA-3'; downstream primer (PUU 286309): 5'-GTG GTC ATC AGG TIC AAT CCC AGT-3'. The expected sizes of the PCR products were 247-bp and 365-bp for the positive control (or viral RNA) and competitor, respectively. RT-PCR reactions were carried out in single tubes using the EZ rTth RNA PCR kit (Perkin-Elmer, Foster City, CA). The PCR reaction consisted of the following: 1X EZ Buffer 50mM bicine, 115 mM potassium acetate, 8% [wt/vol] glycerol [pH 8.2]), 0.3 mM of each dNTP, 5 U of rTth polymerase, 2.5 mM Mn(OAc)₂, and 1 pmol/μl of each primer.

Reverse transcription was carried out at 55°C for 30 min., followed by incubation at 94°C for 2 min. PCR was carried out for 40 cycles as follows: 94°C for 45 sec., 55°C for 45 sec., and 60°C for 45 sec. The final cycle was at 72°C for 7 min. PCR products were resolved on a 2% agarose gel and stained with ethidium bromide. For quantitative RT-PCR, serial dilutions of the competitor negative-stranded RNA (known concentrations in RNA copy numbers) were added to each reaction tube in addition to the unknown vRNA or the positive control RNA. The viral RNA copy number was calculated by comparing the intensity of the viral RNA band...
to that of the competitor band. When the intensities of the two bands are equivalent, the concentration of RNA is equivalent.
CHAPTER III

HUMAN MEMORY CTL RESPONSES TO HTNV INFECTION: IDENTIFICATION OF VIRUS-SPECIFIC AND CROSS-REACTIVE CD8+ EPITOPES ON NUCLEOCAPSID PROTEIN

In our initial studies of human T lymphocyte responses to hantaviruses that cause HFRS, we examined the PBMC from three individuals who had previous laboratory-acquired infections with HTNV. HTNV, the prototype member of the hantavirus genus, is known to cause a relatively severe form of HFRS. These individuals, however, had sub-clinical infections with no evidence of the renal or circulatory abnormalities that are characteristic of HFRS, and no indication of capillary leakage. HTNV infection was confirmed in these subjects by detection of HTNV-specific immunoglobulin in the sera.

The goals of this initial study were to identify the viral proteins that were targeted by T lymphocytes in these individuals, to identify specific CTL epitopes on one or more viral proteins, and to assess whether the CTL responses in these individuals were cross-reactive with other members of the Hantavirus species. In general, little is known about the specific cellular immune responses to hantavirus infection. Our laboratory has previously described several CD8+ and CD4+ N-specific CTL lines that were isolated from the peripheral blood of patients with HPS caused by SNV infection (115). The CD8+ CTL lines isolated in this study were largely SNV-specific, while the CD4+ CTL line was cross-reactive with other hantaviruses. Although recovery from hantavirus infection is thought to confer immunity against the infecting virus, it is not known whether recovery from infection with one hantavirus confers some level of protective immunity against other hantaviruses. Alternatively, it is possible that pre-existing immunity against one hantavirus could predispose an individual to a hyper-activation of the immune system upon infection with a
new hantavirus. This situation could potentially lead to the development of immunopathology. This is an important question, particularly in geographic regions where more than one hantavirus co-circulate (188). An understanding of the characteristics of the T cell response to HTNV, as well as other hantavirus infections, will provide insight into the mechanisms underlying the pathology of hantavirus-induced HFRS and HPS. In a more practical sense, this knowledge may also contribute to the design of effective vaccines and will provide information necessary to construct the tools by which to study the dynamics of the T cell response during acute hantavirus infections.

A. Protein specificity of CTL lines from HTNV-immune donors.

PBMC from three HTNV-immune donors were stimulated in vitro for 7-14 days with an inactivated HTNV preparation. These bulk cultures were then tested for specific lysis of autologous BLCL target cells infected with vaccinia recombinants expressing one or more HTNV proteins. Stimulated PBMC from donor A recognized target cells expressing the nucleocapsid (N) protein, including those infected with vac-N and vac-G1+G2+N (Figure III-1). Lysis of target cells expressing the N protein was relatively low (18-27%) but was consistently higher than killing of target cells infected with wild type vaccinia virus (<7%). PBMC from donor B consistently displayed low-level lysis of target cells expressing the G1 glycoprotein (vac-G1, vac-G1+G2+N) (Figure III-1). Donor B PBMC also displayed low level recognition of the nucleocapsid protein, although the killing of targets expressing G1 was always higher than that of targets expressing N. PBMC from donor C consistently recognized target cells expressing the HTNV N protein (Figure III-1). In summary, the above data show that the PBMC from donors A and C recognized target cells expressing the N protein while PBMC from donor B lysed target cells expressing G1. We were unable to
detect responses specific for the G2 glycoprotein in the PBMC from any of these donors. It is possible that memory T cell responses specific for G2 protein are present in these individuals but are not prevalent enough to detect following short-term stimulation. CTL responses to the G2 protein of SNV have been detected in our laboratory and CD8$^+$ G2-specific CTL lines have been isolated, demonstrating that the SNV G2 protein contains immunogenic epitopes. It is likely that other hantavirus G2 proteins also contain CTL epitopes as well.

When T cell lines were cloned from the PBMC of these donors, the CTL lines isolated from each donor were specific for the protein that was consistently recognized in bulk culture. Results for representative T cell lines isolated from these donors are shown in Table III-1. Both CD8$^+$ and CD4$^+$ T cell lines specific for the N protein were isolated from PBMC from donors A and C. In contrast, only CD4$^+$ T cell lines specific for the G1 glycoprotein were detected in the PBMC from donor B. Although low level recognition of target cells expressing the N protein was seen in the bulk PBMC cultures from donor B, we were not able to isolate N-specific cell lines from this individual. These data demonstrate that HTNV-specific T cell responses are readily detectable in the PBMC of these immune individuals and that responses are directed primarily against the viral N protein and the G1 glycoprotein. The bulk culture responses as well as the specificity and phenotype (CD4/CD8) of the virus-specific T cell lines varied between individuals.
Figure III-1: Bulk culture recognition of HTNV proteins by *in vitro*-stimulated PBMC. Vaccinia viruses used to infect target cells express individual HTNV proteins or a combination of proteins as indicated on the x axis. Lysis of target cells infected with wild-type vaccinia virus served as a negative control. E/T = 80:1. Data from a representative experiment is shown.
Table III-1. Cytotoxicity of CD4⁺ and CD8⁺ T cell lines isolated from HTNV-immune donors

<table>
<thead>
<tr>
<th>Donor</th>
<th>Cell Line</th>
<th>Phenotype</th>
<th>% Specific Lysis</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>vac-G1</td>
<td>vac-G2</td>
</tr>
<tr>
<td>A</td>
<td>1-C8</td>
<td>CD8</td>
<td>-2.2</td>
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<tr>
<td>A</td>
<td>3-G7</td>
<td>CD8</td>
<td>-2.6</td>
</tr>
<tr>
<td>A</td>
<td>10-E2</td>
<td>CD8</td>
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</tr>
<tr>
<td>A</td>
<td>10-B5</td>
<td>CD8</td>
<td>-1.6</td>
</tr>
<tr>
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</tr>
<tr>
<td>C</td>
<td>3-F8</td>
<td>CD4</td>
<td>-3.6</td>
</tr>
</tbody>
</table>

Data from representative cell lines are shown. E/T ratios vary from 20:1 to 50:1.

Cell lines were >95% CD4 or CD8 by flow cytometry.

Specific lysis values >10% above background were considered positive and are underlined.

(-) Target not tested.
B. Peptide specificity of CD8\(^+\) CTL lines from HTNV-immune donors A and C.

To identify the specific peptide on the N protein recognized by the CD8\(^+\) CTL lines, each cell line was tested in \(^{51}\)Cr release assays against target cells pulsed with a series of seventy overlapping peptides spanning the entire HTNV nucleocapsid protein. The 15-mer peptides recognized by the CD8\(^+\) CTL lines of the two donors are shown in Table III-2. Interestingly, all N-specific CD8\(^+\) CTL lines isolated from donors A and C recognized one of two 15-mer peptides on the nucleocapsid protein. CTL lines 3-G7 and 10-E2 from donor A and 3A-G3 from donor C recognized target cells pulsed with a peptide that is located at the amino terminus of the N protein and spans amino acids 7-21 (LQREINAHEGQLVIA). CTL line 1-C8 from donor A and lines 1A-B7, 3A-B8, 3A-C4 and 3A-C10 from donor C recognized target cells pulsed with a peptide that is located at the extreme carboxy terminus of the protein and spans amino acids 416-429 (VKVKEISNQEPLKL). Subsequent screening of numerous other cell lines from these two donors resulted in the isolation of additional CTL lines that recognized the same peptides (data not shown).

Once the nucleocapsid peptides (15-mers) recognized by the CTL lines were identified, sequential N- and C-terminal truncations of the peptides were synthesized and tested in \(^{51}\)Cr release assays. The patterns of peptide recognition of representative clones from each donor are shown in Figures III-2 and III-3. The clones specific for N peptide 7-21 (donor A: 3-G7, 10-E2; donor C: 3A-G3) were all found to recognize a minimal 9-mer peptide spanning amino acids 12-20 (NAHEGQLVI). A representative CTL line from each donor is shown in Figure III-2A&C. These cell lines required comparable peptide concentrations for target cell recognition (Figure III-3A), suggesting that the TCRs expressed by these cell lines have comparable affinity for the peptide/MHC complex. The CTL lines specific for the N peptide 416-429 (donor A: 1-C8; donor C: 1A-B7, 3A-B8, 3A-C4, 3A-C10)
were all found to recognize a minimal 9 amino acid epitope at the extreme carboxy terminus of the protein spanning amino acids 421-429 (ISNQEPPLKL). Data from representative CTL lines from both donors are shown in Figure III-2B&D. These CTL lines also required similar peptide concentrations for target cell recognition with the exception of CTL lines 1A-B7 and 3A-C4, which required a slightly higher peptide concentration for optimal target cell recognition (Figure III-3B).

Table III-2. Nucleocapsid peptide recognition of CD8⁺ CTL lines

<table>
<thead>
<tr>
<th>Donor A CTL Line</th>
<th>Amino Acids</th>
</tr>
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<tbody>
<tr>
<td>1-C8</td>
<td>416-429</td>
</tr>
<tr>
<td>3-G7</td>
<td>7-21</td>
</tr>
<tr>
<td>10-E2</td>
<td>7-21</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Donor C CTL Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A-B7</td>
</tr>
<tr>
<td>3A-B8</td>
</tr>
<tr>
<td>3A-C4</td>
</tr>
<tr>
<td>3A-C10</td>
</tr>
<tr>
<td>3A-G3</td>
</tr>
</tbody>
</table>
Figure III-2: Localization of CTL epitopes on HTNV N protein recognized by representative CTL lines from donors A and C. Target cells were autologous BLCLs either infected with vac-N or wild-type vac or pulsed with peptide (25 μg/ml). E/T = 10:1.
Figure 111: Peptide dose-response curves for N peptide-specific CTL lines from Donors A and C. Autologous BLCLs were pulsed with peptide at the indicated concentrations. (A) CTL lines specific for N₁₂₋₂₀. (B) CTL lines specific for N₄₂₁₋₄₂₉. Data from a representative experiment is shown. E/T = 10:1.
C. HLA Restriction of N-specific CD8+ CTL lines.

HLA restriction of the CD8+ CTL lines was determined by testing each CTL line against a panel of partially HLA-matched allogeneic BLCL infected with a vaccinia recombinant expressing the HTNV N protein. To ensure that lysis of the allogeneic targets was not due to recognition of an allogeneic or vaccinia determinant, each target cell line was also infected with WT vaccinia virus and tested in the same assay. The results of the HLA restriction analysis of representative CTL lines isolated from donors A and C are shown in Figure III-4.

All of the CD8+ CTL lines generated from donor A recognized and lysed allogeneic target cells expressing both HLA A1 and B51 (Figure III-4A&B and data not shown). CTL lines 3-G7 and 10-E2, which recognized N_{12-20}, also recognized an Hmy-2C1R cell line expressing only HLA B51, indicating that these CTL lines are restricted by HLA B51 (Figure III-4A and data not shown). CTL line 1-C8, which recognized N_{421-429}, did not recognize the Hmy-B51 target cells. Since this cell line also recognized target cells expressing A1 alone, it must be restricted by HLA A1 (Figure III-4B).

Donor C CTL line 3A-G3, which is specific for N_{12-20}, recognized only target cells that express B51, including the Hmy-2C1R-B51 cell line (Figure III-4C), which definitively demonstrates that CTL line 3A-G3 is restricted by B51. CTL lines 1A-B7, 3A-B8, 3A-C4 and 3A-C10 from donor C, which are all specific for N_{421-429}, recognized allogeneic target cells expressing both A1 and All, A1 and B8 and A1 and B51 (Figure III-4D and data not shown) but did recognize other targets expressing All, B8 or B51, indicating that these cell lines are all restricted by HLA A1.

The B51-restricted epitope N_{12-20} [NAHEGQLVI] conforms to the consensus HLA B51 binding motif with an Ala at position 2 (A, P or G preferred) and an Ile at position 9 (I or V preferred). The A1-binding peptide N_{421-429} [ISNQEPLKL] conforms poorly to the HLA
A1 binding motif, which contains a glutamine or glutamic acid at position 3 and an aromatic tyrosine at position 9 (189).

In summary, CTL lines from both donors that recognized the N-terminal nucleocapsid epitope (N_{12-20}) were restricted by HLA B51 while CTL lines that recognized the C-terminal nucleocapsid epitope (N_{421-429}) were restricted by HLA A1.
Figure III-4: HLA restriction of CTL lines from donors A and C. CTL lines were tested in 51Cr-release assays against a panel of partially HLA-matched allogeneic target cells infected with vac-N. The HLA alleles shared by the donor (autologous) and the allogeneic target cells are shown on the y axis. Data from representative experiments are shown. E/T = 10:1.
D. Cross-reactivity of CTL lines isolated from HTNV-immune donors

Little is known about cross-reactive immunity between different hantaviruses. The hantavirus N proteins have an overall sequence identity of 50%, although specific regions of the protein are more highly conserved (e.g. the C-terminal 100 amino acids are 85% identical). Closely related hantaviruses, such as HTNV and SEOV, have nucleocapsid proteins that are 82% identical. The high level of sequence identity suggests that hantavirus infection may result in the development of immune responses that are cross-reactive among numerous hantaviruses. To test whether the CTL lines were cross-reactive with other hantaviruses, we tested the CTL lines for recognition of target cells infected with a recombinant vaccinia virus expressing the SNV N protein. The CTL lines were also tested against targets pulsed with corresponding 9-mer peptide epitopes from various other hantaviruses, which were synthesized based on published sequences.

The CD8+ CTL lines that recognized the N-terminal epitope N\textsubscript{12-20} (donor A:3-G7, 10-E2; donor C:3A-G3) did not recognize target cells infected with a recombinant vaccinia virus expressing the SNV N protein or target cells pulsed with 9-mer peptides representing the corresponding epitope from various other hantaviruses including SNV, DOBV, PHV, ANDV and PUUV (Figure III-5& III-6A). This epitope is variable among the different hantaviruses, differing by up to 4 amino acids (Figure 6A). The DOBV peptide varies by only one amino acid (A2→N) from the HTNV epitope, but was not recognized by any of the CTL lines (Figure III-6). This lack of recognition may be the result of the loss of peptide binding to the HLA B51 molecule, since the amino acid change in the epitope derived from DOBV occurs at an anchor position. However, we have not performed peptide binding studies to formally demonstrate this. The SEOV peptide also varies by only one amino acid from the HTNV
epitope (N1→S); it was recognized by CTL lines 3-G7 and 3A-G3 but not by 10-E2 (Figure III-6A).

The CTL lines specific for the C-terminal epitope N\textsubscript{421-429} (Donor A: 1-C8, Donor C: 1A-B7, 3A-B8, 3A-C4 and 3A-C10) all recognized target cells infected with a vaccinia recombinant expressing the SNV N protein (Figure III-5). It is not surprising that these CTL lines are cross-reactive with the SNV nucleocapsid since the epitope is identical between the two viruses (ISNQEPLKL). This result demonstrated that the epitope is processed and presented in the context of infection with a recombinant vaccinia virus expressing either HTNV or SNV N. This epitope is also identical in other hantaviruses including ANDV, NYV, DOBV and SNV strain CC107. The corresponding C-terminal epitope in PUUV, PHV, and Bayou virus (BAYV) differs by one amino acid with a conservative Leu to Ile change at position 9. Four of the five CTL lines isolated recognized this epitope: Donor A: 1-C8 and Donor C: 1A-B7, 3A-B8, 3A-C10 (Figure III-6B and data not shown). The epitope in SEOV also differs from the HTNV epitope by one amino acid with a Leu to Met change at position 7. Three of the five CTL lines lysed target cells pulsed with this peptide; cell lines 1A-B7 and 3A-C4 failed to recognize and lyse these target cells (Figure III-6B and data not shown). The differences between the CTL lines in recognition of the peptides derived from the various hantaviruses indicate that these CTL lines are heterogeneous and are not likely to have resulted from the monoclonal expansion of a single dominant clone.
Figure III-5: Recognition of SNV or HTNV N protein by HTNV-specific CD8+ CTL lines from Donors A and C. Autologous BLCLs were infected with recombinant vaccinia viruses expressing either HTNV N or SNV N. BLCLs infected with wild-type vaccinia virus were used as a negative control. E/T = 10:1.
Figure III-6: Cross-reactivity of representative CD8+ CTL lines from Donors A and C against corresponding epitopes from several hantavirus N proteins. CTL lines were tested against autologous BLCLs pulsed with the indicated epitopes at 25 μg/ml. Amino acids that differ from the corresponding amino acid in the HTNV peptide are underlined. E/T ratio = 10:1. (A) CTL lines specific for N_{12-20}. (B) CTL lines specific for N_{421-429}. 
E. Recognition of HTNV N epitopes in 7-day bulk culture

The HTNV-specific clones isolated from donors A and C are all specific for one of two epitopes on the nucleocapsid protein. Both epitopes are located at the termini of the protein, potentially allowing them to be more rapidly degraded and more efficiently presented on MHC class I molecules than more internally located peptides. In order to address the in vivo significance of these epitopes in Donor C, we tested recognition of these epitopes in bulk cultures after short-term stimulation with inactivated HTNV. Seven day bulk cultures of donor C PBMC demonstrated significant recognition of both peptide epitopes, with the N-terminal epitope (N12-20) being recognized at levels comparable to that of the whole N protein (vac-N) (Figure III-7). Frequency analysis confirms that T cells recognizing these two epitopes are present at relatively high frequencies in the peripheral T cell pool. Single cell IFN-γ secretion analysis (ELISPOT) of donor C PBMC indicates that T cells specific for N12-20 have a frequency ranging from 1/7,326-1/8,968 PBMC, and T cells specific for N421-429 have a frequency ranging from 1/18,181-1/19,417 PBMC. These frequencies are comparable to those detected for several immunodominant epitopes from influenza virus (187, 190).
Figure III-7: Recognition of HTNV proteins and identified CTL epitopes following short-term in vitro stimulation. PBMC (2-5 X 10^6) were stimulated with γ-irradiated HTNV for 7 days. The bulk culture was tested on day 7 against autologous BLCLs either infected with recombinant vaccinia viruses expressing HTNV proteins or pulsed with the indicated peptides (25 μg/ml). E/T ratio = 80:1.
CHAPTER IV

HIGH LEVELS OF HUMAN MEMORY CTL FOLLOWING CLINICAL PUUV INFECTION: DOMINANT AND POLYCLONAL CTL RESPONSES TO THE NUCLEOCAPSID PROTEIN

Initial studies of memory T lymphocyte responses in individuals who had subclinical, laboratory-acquired infections with HTNV provided the first demonstration of CTL responses to viral proteins and description of CTL epitopes on the virus. We were also interested in studying memory T cell responses in individuals who had HFRS as a result of natural exposure to the virus. To study memory T cell responses following the resolution of HFRS acquired naturally, we studied a group of 13 Finnish individuals who had clinical infections with PUUV between the years 1984 and 1995 (Table IV-1). These subjects were all hospitalized with HFRS, but had varying degrees of disease severity.

The goals of this study were as follows: 1.) Identify the viral proteins targeted by the CTL response following PUUV infection; 2.) Identify and characterize CTL epitopes on PUUV; 3.) Assess whether the CTL responses detected in these individuals were cross-reactive with other hantaviruses; and 4.) Quantitate the PUUV epitope-specific CTL precursor frequencies in all the donors.

A. Dominance of PUUV N protein as target of memory CTL during HFRS

PBMC from eleven donors who had HFRS between the years 1984 and 1995 were stimulated \textit{in vitro} for 7 to 8 days with live PUUV. Following \textit{in vitro} stimulation, bulk cultures were screened for recognition of autologous BLCLs infected with recombinant vaccinia viruses expressing PUUV proteins. CTL responses against one or more PUUV proteins were detectable in PBMC from 7/11 donors. CTL activity was directed against the N protein in 6 of the cultures (donors 1-6), against the G1 glycoprotein in 5 (donors 1,3,5,6 &
Following re-stimulation with PUUV and autologous, irradiated PBMC, we were able to detect CTL responses against N protein in bulk cultures from two of the donors in which virus-specific responses were undetectable at day 7-8 (data not shown). High levels of vaccinia virus (vac)-specific “background” CTL responses were detected in the bulk cultures from three donors (donors 4, 5 & 7), despite the inclusion of unlabeled vac-infected target cells in the assays. The specific lysis of wild-type (WT) vaccinia virus-infected targets in these cultures was 26%, 18% and 29%, respectively. However, the lysis of target cells infected with recombinant viruses expressing PUUV proteins was significantly higher than that of target cells infected with wild-type vaccinia virus. Vac-specific responses in all other donors were less than 5%. The specific lysis of target cells infected with vaccinia virus recombinants expressing PUUV proteins was considered to be positive if it was greater than 10% above that of WT vaccinia virus-infected targets.

PUUV-specific CTL cell lines were identified and characterized by in vitro expansion and cloning of bulk cultures from donors 1 and 2. Forty-three PUUV-specific cell lines were isolated from donor 1; of these 42 were N-specific and 1 was G1-specific. Twenty cell lines were isolated from donor 2; of these 19 were N-specific and 1 was G2-specific. The predominance of cell lines specific for N suggests that the N protein may be the dominant target of the T cell response during PUUV infections. The fact that we were only able to identify one G1-specific cell lines from donor 1 was somewhat surprising, since the bulk culture from this donor showed comparable levels of N- and G1-specific CTL activity. We did not detect bulk culture CTL specific for G2 in either of these donors. However, the fact that G2-specific CTL responses were detected in bulk cultures from two other donors (Figure IV-1), and the recovery of a G2-specific CTL line from donor 2, indicates that these cells were present at detectable levels in some individuals.
Table IV-1. Characteristics of PUUV-immune donors.

<table>
<thead>
<tr>
<th>Donor #</th>
<th>Current Age (yrs)</th>
<th>Year of Illness</th>
<th>HLA Class I Alleles</th>
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<td></td>
<td></td>
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</tr>
<tr>
<td>13</td>
<td>62</td>
<td>1984</td>
<td>1, 2</td>
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Figure IV-1: Bulk culture recognition of PUUV proteins. PBMC (4-5X10^6) were stimulated with 20 µl of infectious PUUV (strain K27) and cytotoxicity assays were performed using autologous BLCLs infected with recombinant vaccinia viruses expressing PUUV N, G1, G2/Bs or G2/Sm protein as targets. The recombinant vaccinia virus designated vac-G2/Bs contains the first half of the G2 cDNA, encoding amino acids 1 to 256, and the recombinant designated vac-G2/Sm contains the second half of the G2 protein, encoding amino acids 227 to the end of the protein. E/T ratios were 60:1 or 80:1. Unlabeled wild-type vaccinia virus infected BLCL were included in all wells at a ratio of 10:1 unlabeled: labeled targets, to reduce levels of vac-specific lysis. Data shown represent the percent specific lysis after subtraction of wild-type vaccinia virus background lysis. Responses greater than 10% above background were considered positive.
B. Broad CTL responses to PUUV N protein in two individuals

Nucleocapsid-specific CTL isolated from donors 1 and 2 were characterized using a panel of overlapping 15-mer peptides that span the PUUV N sequence. Each CTL line isolated recognized target cells pulsed with only one or two (adjacent) of the 70 overlapping peptides that span the N protein (data not shown). Optimal CTL epitopes were then defined by testing each CTL line against sequential N- and C-terminal truncations of the appropriate 15-mer peptide. The restricting HLA alleles required for peptide recognition by these CTL lines was determined using a panel of partially HLA-matched BLCLs as targets in \(^{51}\)Cr-release assays (data not shown). A summary of the epitopes and representative CTL lines identified in these donors is shown in Table IV-2.

CD8\(^{+}\) CTL lines from donor 1 were found to recognize one of four epitopes: 1 restricted by A2 (N\(_{204-12}\)), 1 restricted by B7 (N\(_{173-81}\)) and 2 restricted by B8 (N\(_{173-81}\) and N\(_{243-51}\)). CD4\(^{+}\) CTL lines were also isolated from this donor which recognized N\(_{239-50}\). The restricting allele for these CD4\(^{+}\) CTL lines has yet to be determined. Figure IV-3 demonstrates the cytolytic activity of the CD8\(^{+}\) CTL lines using target cells pulsed with increasing dilutions of the optimal peptide epitopes.

The CTL clones specific for N\(_{204-12}\) appeared to have a range of affinities for the peptide/MHC complex, as reflected by the wide range of peptide concentrations required for sensitization of target cells (Figure IV-3A). This peptide (GLFPTQIQV) conforms to the defined HLA binding motif for A2, with the preferred leucine at position 2 and valine at position 9.

The CTL lines specific for N\(_{173-81}\) displayed more uniform peptide dose-response curves, suggesting that their TCRs have similar affinities for the peptide-MHC complex (Figure IV-3B). It is interesting to note, however, that we identified both B7-restricted (3-C8 and 10-F5) and B8-restricted (10-G4) CTL lines that recognized the same epitope (Table IV-2).
and Figure IV-2). This peptide (RPKHLYVSM) conforms to the defined HLA B7 motif reasonably well, with the preferred proline at position 2 and a methionine at position 9 (L or F preferred); and to the B8 motif less well, with the preferred lysine at position 3 but a leucine at position 5 (K or R preferred).

CD8+ CTL from donor 2 were also found to recognize one of four epitopes: 1 restricted by HLA A2 (N204-12), 1 restricted by A28 (N164-78) and 2 (N236-50 and N212-20) for which the HLA restriction has yet to be determined. CTL lines specific for the latter two epitopes were tested against a large panel of partially HLA-matched target cells, including cells matched at each of the HLA A and B alleles (Table IV-1). CTL lines specific for these epitopes recognized only a single allogeneic target cell line that shares A2, A28 and B44. However, these CTL lines failed to recognize numerous other target cell lines sharing these alleles, as well as those sharing HLA B8 (data not shown). The data suggest that the restricting allele used by these CTL lines is likely to be an HLA Cw allele.

The cytolytic activity of CTL lines specific for N236-50, N212-20 and N164-78 is shown in Figure IV-4. The CTL lines specific for N236-50 required very high peptide concentrations for sensitization of target cells (Figure IV-4A). The length of this peptide (15 amino acids) is probably sub-optimal for binding to the class I HLA molecule and this may account for the absence of target cell recognition at lower peptide concentrations. However, single amino acid deletions from either the N- or C-terminal end of this peptide abolished recognition by these CTL lines, suggesting that the entire 15-mer is required for recognition (data not shown).

The two CTL lines specific for N212-20 appeared to have comparable affinities for the peptide/MHC complex, as reflected by the similar peptide dose-response curves (Figure IV-4B). The CTL line 3-B8 was tested against a 15-mer peptide (N164-78) and required lower
peptide concentrations for target cell sensitization than required by the CTL lines specific for N_{236-50}.
<table>
<thead>
<tr>
<th>Donor</th>
<th>CD8/CD4</th>
<th>HLA Restriction</th>
<th>Amino Acids</th>
<th>CTL Lines</th>
</tr>
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<tbody>
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<td>204-212</td>
<td>1A-C11</td>
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Figure IV-2: HLA restriction of N_{173-81}-specific CTL lines isolated from donor 1. Autologous BLCL or Hmy.CIR cell lines expressing the indicated HLA allele were infected with a recombinant vaccinia virus expressing PUUV N and used as targets in a ^{51}Cr-release cytotoxicity assay. E/T=10.
Figure IV-3. Peptide dose-response curves of CD8+ N-specific CTL lines isolated from donor 1. A) CTL lines specific for the A2 epitope N204-12. B) CTL lines specific for the B7,B8 epitope N173-81. C) CTL line specific for the B8 epitope N243-51. Lysis of targets not pulsed with peptide was negative in all assays.
Figure IV-4. Peptide dose-response curves for CD8⁺ N-specific CTL lines isolated from donor 2. A) CTL lines specific for N_{236-50}. B) CTL lines specific for N_{212-20}. C) CTL line 3-B8, specific for the A28-restricted epitope N_{164-78}.
C. High frequencies of PUUV-specific CTL in PUUV-immune individuals

In order to determine whether the epitopes identified in this study are determinants that are commonly targeted by PUUV-specific CTL, we studied a panel of PBMC from 13 PUUV-immune donors, including the 7 donor PBMC in which PUUV-specific responses were detected in bulk cultures (Figure IV-1). Each of the 13 individuals expresses one or more of the restricting HLA alleles required by the CTL clones isolated from donors 1 and 2 (Table IV-1).

Of the 13 individuals tested, 8 had detectable PUUV-specific T cell responses in 16-18 hour IFN-γ ELISPOT assays (Figure IV-5). Three individuals had high frequencies of epitope-specific T cells in their PBMC (donors 1, 11 & 13; Figure IV-5A), with some epitope-specific T cells present at frequencies as high as 300 per 10^6 PBMC. The hierarchy of epitope recognition varied from donor to donor, with different epitope-specific responses dominating in different individuals. The N173-81 response dominated in donor 1, the N243-51 response in donor 11, and the N204-12 response dominated in donor 13.

Five additional donors (donors 2, 6, 7, 9 & 10) had somewhat lower responses to these epitopes (Figure IV-5B). The frequency of epitope-specific T cells seen in these donors was between 13 and 61/10^6 PBMC. These responses are comparable to frequencies of many commonly recognized influenza virus epitopes (187, 190). In fact, when PBMC from the 3 A2+ donors in this study were stimulated with the influenza A epitope M158-66 in ELISPOT assays, none of the donors had detectable responses (Table IV-3). In these donors, the hierarchy of responses also varied between individuals.

A summary of the range of precursor frequencies seen in these donors is shown in Table IV-3. Of the three epitopes to which T cell responses were detected, the A2-restricted epitope (N204-12) was recognized most consistently, with detectable responses seen in all A2+
donors tested (3/3). The B8-restricted epitope N\textsubscript{243-51} was recognized in 8 of 12 donors tested, and the N\textsubscript{173-81} (B7 or B8 restricted) epitope was recognized in 2 of 12 donors. We did not detect IFN-\(\gamma\) responses to the N\textsubscript{164-78} epitope in any of the A28\(^+\) donors tested (3/3), including the donor from whom the N\textsubscript{164-78}-specific clone was isolated (donor 2). Since we have not identified the optimal epitope recognized by this CTL, we used a 15-mer peptide to stimulate the PBMC in the ELISPOT assays. It is possible that this peptide is not optimal for stimulation of epitope-specific cells and this may have contributed to our inability to detect a response in the donors expressing A28. It is also possible that the frequencies of T cells specific for this epitope were below the limit of detection.

These data demonstrate that PUUV N-specific memory CTL are abundant in the peripheral blood of immune individuals for many years after acute infection, and that detection of these memory CTL populations does not require \textit{in vitro} expansion.

We did not detect responses to any of the 4 defined CD8\(^+\) epitopes in the PBMC of the other 5 donors (donors 3, 4, 5, 8 & 12). It is clear that virus-specific memory T cells are present in the PBMC of these donors, since N-specific CTL activity was detectable in at least 3 of these donors’ PBMC (donors 3, 4 & 5) following 7 days of \textit{in vitro} stimulation with infectious PUUV (Figure IV-1). It is likely that there are other CD8\(^+\) epitopes on the N protein that have not yet been identified, and that CTL with distinct specificities contribute to the CTL response in these donors. We have also demonstrated proliferation responses to inactivated PUUV in these donors (data not shown), further indicating that all donors possess virus-specific memory T cells.
Figure IV-5. Detection of virus-specific CD8+ memory T cells by IFN-γ ELISPOT. PBMC were stimulated with 10 μg/ml of the indicated peptides in a 16-18 hour assay. Input cell numbers ranged from 2-4x10⁶/well. Peptide stimulations were performed in triplicate wells. PBMC stimulated with a B8-restricted epitope from Hepatitis C virus NS3 (NS31402-1411) were included for comparison with PUUV-specific B8-restricted epitopes. PBMC incubated with medium alone were included as negative controls. The data are presented as # of IFN-γ producing cells/10⁶ PBMC, with medium control values subtracted. The number of spots in the medium control wells ranged from 0 to 5. All ELISPOT experiments were performed at least twice and mean values are shown. A) PUUV-immune individuals who had high precursor frequencies (>100/10⁶ PBMC) of CTL specific for one or more CD8+ epitopes on PUUV N. B) PUUV-immune individuals who had lower precursor frequencies (10-100/10⁶ PBMC) of CTL specific for one or more CD8+ epitopes on PUUV N.
Table IV-3. Recognition of CD8+ PUUV epitopes in PBMC from immune donors.

<table>
<thead>
<tr>
<th>Epitope</th>
<th>HLA restriction</th>
<th># of donors tested*</th>
<th># of responders</th>
<th>Range of IFN-γ producing T cells/10^6 PBMC</th>
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</thead>
<tbody>
<tr>
<td>N173-81</td>
<td>B7, B8</td>
<td>12</td>
<td>2</td>
<td>52-304</td>
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<td>N243-51</td>
<td>B8</td>
<td>12</td>
<td>8</td>
<td>13-188</td>
</tr>
<tr>
<td>N204-12</td>
<td>A2</td>
<td>3</td>
<td>3</td>
<td>16-200</td>
</tr>
<tr>
<td>N164-78</td>
<td>A28</td>
<td>3</td>
<td>0</td>
<td>(-)</td>
</tr>
<tr>
<td>M731-39</td>
<td>A24</td>
<td>3</td>
<td>0</td>
<td>(-)</td>
</tr>
<tr>
<td>Flu M158-66</td>
<td>A2</td>
<td>3</td>
<td>0</td>
<td>(-)</td>
</tr>
</tbody>
</table>

*Only donors possessing the indicated HLA allele were tested.
D. Cross-reactivity of CTL lines isolated from donors 1 and 2

We were interested in learning whether the CTL responses generated against one hantavirus also recognized the corresponding epitopes from other hantaviruses, thus potentially conferring cross-protective immunity. We therefore tested the CTL lines isolated from donors 1 and 2 against autologous BLCLs infected with recombinant vaccinia viruses that express either PUUV, HTNV or SNV N proteins. As shown in Table IV-4, the CTL lines specific for N_{202-12} failed to recognize the N protein from either HTNV or SNV. The sequences of HTNV and SNV N proteins both differ from the PUUV sequence at this epitope by 4 amino acids, including a V to A change at the anchor position 9 (Table IV-5). Of the two B7-restricted and one B8-restricted CTL lines that recognized PUUV N_{173-81}, only 10-F5 displayed cross-reactive recognition of the HTNV N protein. This epitope is fairly well conserved among the viruses; both the HTNV and SNV sequences at this epitope differ from the PUUV epitope by only two amino acids, and in both the preferred P at position 2 is conserved (Table IV-5).

The CD8+ CTL lines from donor 2 that recognize PUUV N_{236-50} are also primarily PUUV-specific with most displaying no recognition of targets expressing either the HTNV or SNV N protein. However one CTL line, 1B-G9, consistently recognized targets expressing the SNV N protein as well as those expressing the PUUV N protein (Table IV-4). The sequence at this epitope is extremely variable among different hantaviruses (Table IV-5), and it is thus surprising that this CTL line was able to recognize the corresponding sequence from the SNV N protein.

Neither of the cell lines that recognize PUUV N_{212-20} were cross-reactive with HTNV or SNV N protein, despite the relatively high level of sequence conservation at this epitope (Table IV-5). However, since we have not yet determined the HLA restriction of these CTL lines, we cannot accurately predict which residues within the epitope might be critical for
recognition of the peptide-HLA complex by the TCR, or for binding of this peptide to the HLA molecule. Finally, the CTL line that recognizes \( N_{164-78} \) (3-B8) is cross-reactive with the HTNV N protein, but does not recognize the SNV N protein. This epitope is relatively well conserved between PUUV and HTNV virus, with the corresponding epitope on HTNV N differing by 3 amino acids, and less well conserved between PUUV and SNV viruses, which differ by 6 amino acids at this epitope.

In summary, of 22 CTL lines tested for cross-reactivity against HTNV and SNV viruses only 3 CTL lines showed cross-reactive lysis, suggesting that the CTL responses identified in this study were highly PUUV-specific. None of these cross-reactive CTL lines recognized the N protein from both HTNV and SNV viruses, despite the fact that some of the epitopes share considerable sequence identity among the different hantaviruses.
Table IV-4. Cross-reactivity of PUUV N-specific CD8+ CTL lines.

<table>
<thead>
<tr>
<th>Donor</th>
<th>CTL Line</th>
<th>Epitope</th>
<th>PUUV N</th>
<th>HTNV N</th>
<th>SNV N</th>
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<td>-1.1</td>
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<td></td>
<td>1A-D10</td>
<td>N204-12</td>
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<td>-1.2</td>
<td>-3.3</td>
</tr>
<tr>
<td></td>
<td>1A-E5</td>
<td>N204-12</td>
<td>49.6</td>
<td>-5.6</td>
<td>-3.8</td>
</tr>
<tr>
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<td>1B-C3</td>
<td>N204-12</td>
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<td>-1.7</td>
<td>-1.4</td>
</tr>
<tr>
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<td>3-C3</td>
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<td>-3.9</td>
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<tr>
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<td>3-D10</td>
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<tr>
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<td>-6.9</td>
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</tr>
<tr>
<td></td>
<td>3-E11</td>
<td>N204-12</td>
<td>43.4</td>
<td>-3.0</td>
<td>-6.7</td>
</tr>
<tr>
<td></td>
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<td>N204-12</td>
<td>37.6</td>
<td>-2.8</td>
<td>-3.9</td>
</tr>
<tr>
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<td>-3.5</td>
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<td>N212-20</td>
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<td>0.4</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>3-B8</td>
<td>N164-78</td>
<td>27.4</td>
<td>25.9</td>
<td>-3.0</td>
</tr>
</tbody>
</table>

*E/T ratio = 10.

Specific lysis values >10% above background were considered positive and are underlined.
Table IV-5: Sequence comparison of epitopes between PUUV, HTNV and SNV viruses.

<table>
<thead>
<tr>
<th></th>
<th>PUUV</th>
<th>HTNV</th>
<th>SNV</th>
</tr>
</thead>
<tbody>
<tr>
<td>N204-12</td>
<td>G L F P T Q I Q V</td>
<td>G L Y P A Q I K A</td>
<td>G L F P A Q V K A</td>
</tr>
<tr>
<td>N212-20</td>
<td>V R N I M S P V M</td>
<td>A R Q M I S P V M</td>
<td>A R N I I S P V M</td>
</tr>
</tbody>
</table>
E. Heterogeneity of CD8+ CTL clones specific for N\textsubscript{204-12}

Since we identified numerous CTL lines from donor 1 that were specific for the same peptide/HLA combination, we were interested in determining whether this population was polyclonal. To assess the diversity of the N\textsubscript{204-12}-specific T cell clones isolated from donor 1, we identified the TCR Vβ chains expressed by these clones using PCR amplification. A single PCR product was generated for each clone, indicating the expression of a single TCR Vβ chain (data not shown). A summary of the TCR Vβ chains expressed by 9 CTL clones specific for N\textsubscript{204-12} is shown in Table IV-6.

Among the 9 CTL clones analyzed, we found 5 different Vβ chains that were expressed, including Vβ 2, 3, 5.2, 8 and 13.1. Three of the CTL clones expressed Vβ13.1, and three expressed Vβ5.2, suggesting that these Vβ chains may be preferentially used by CTL specific for this epitope. Further phenotypic analysis, including Vα chain usage and CDR3 sequencing, will be required to determine if the clones that express the same Vβ chains are distinct from one another, or if they represent the progeny of a single precursor clone. The fact that 5 different TCR Vβ chains were used by 9 CTL clones suggests that the TCRs expressed by this CTL population are quite diverse and that multiple TCR rearrangement events occurred to produce CTL clones that recognize the identical peptide.
Table IV-6: Summary of TCR Vβ chains expressed by N204-12-specific T cell clones isolated from donor 1.

<table>
<thead>
<tr>
<th>Clone</th>
<th>TCR Vβ</th>
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<tr>
<td>1A-C11</td>
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</tr>
<tr>
<td>1A-E5</td>
<td>8</td>
</tr>
<tr>
<td>1B-C3</td>
<td>2</td>
</tr>
<tr>
<td>3-C3</td>
<td>5.2</td>
</tr>
<tr>
<td>3-D10</td>
<td>13.1</td>
</tr>
<tr>
<td>3-E4</td>
<td>5.2</td>
</tr>
<tr>
<td>10-B10</td>
<td>3</td>
</tr>
</tbody>
</table>
F. Isolation and characterization of a PUUV G2-specific CD8+ CTL clone

In addition to isolating numerous virus-specific CTL lines that were specific for the PUUV N protein, we were able to isolate and characterize one CTL clone that was specific for the PUUV G2 glycoprotein from donor 3. This CTL clone was derived from a bulk culture of PBMC that had been stimulated with inactive (γ-irradiated) PUU K27 virus (kindly provided by Connie S. Schmaljohn), and was the only CTL line that was successfully isolated and maintained from this culture.

This clone was later shown to reproducibly recognize targets infected with the vac-PUU G2/Bs and vac-SNV G2 recombinants (Figure IV-6 & data not shown). In fact, the recognition of target cells infected with vac-SNV G2 was consistently higher than that of target cells infected with vac-PUU G2/Bs (Figure IV-6). This is likely to be due to the relatively low level of G2 protein expression from the recombinant vaccinia virus expressing the PUUV G2 protein (Terajima, Van Epps et al, manuscript in preparation). Since the recognition of the vac-SNV G2 construct was superior to that of the vac-PUU G2/Bs construct, we used this recombinant virus, as well as overlapping peptides derived from SNV G2, to further characterize the clone.

HLA restriction of clone 1A-E2 was determined by screening a panel of partially HLA-matched allogeneic BLCL infected with vac-SNV G2 as target cells in cytotoxicity assays. These experiments clearly demonstrated that the clone was restricted by HLA A24, since this was the only HLA allele that was shared by all the target cells recognized by the clone (Figure IV-7).
Figure IV-6: Recognition of hantavirus G2 glycoproteins from PUUV, SNV and HTNV viruses by CTL clone 1A-E2 from donor 3. The clone was tested in $^{51}$Cr-release cytotoxicity assays for recognition of autologous BLCLs infected with vaccinia virus recombinants expressing PUU G2/Bs, SNV G2 or HTNV G2. E/T=10.

Figure IV-7: HLA restriction of CTL clone 1A-E2 from donor 3. The clone was tested in $^{51}$Cr-release cytotoxicity assays against a panel of partially HLA-matched allogeneic target cells infected with vac-SNV G2. The HLA alleles shared by donor 3 (autologous) and the allogeneic target cells are shown on the y axis. E/T=10.
To identify the precise epitope within the G2 protein that was recognized, we tested the clone in cytotoxicity assays against a panel of overlapping 20-mer peptides spanning the first half of the SNV G2 protein (data not shown). This analysis revealed that a single 20-mer peptide which spanned amino acids 747-766 (HAEIQNLGHWMGDGTFNIKTA) on the SNV M segment was capable of sensitizing target cells for lysis by the 1A-E2 clone. The sequence of this region differed between SNV and PUUV G2 proteins by 3 amino acids (PUUV M723-742: HAEIQHLGHWMDATFNLKTA). Sequential C- and N-terminal truncations of the PUUV M723-742 peptide showed that the minimal peptide required for sensitization of target cells for lysis by the 1A-E2 clone was a 9-mer peptide spanning amino acids 731-739 (Figure IV-8). Target cells pulsed with peptides M727-38 and M725-36 were recognized and lysed, but levels of lysis of these targets was considerably lower than those pulsed with the M731-39 peptide. Target cells were sensitized for lysis by the clone with peptide concentrations as low as 25 nM of peptide M731-39 (Figure IV-9).
Figure IV-8: Localization of the minimal peptide epitope on PUUV G2 protein recognized by clone 1A-E2 from donor 3. Target cells were autologous BLCLs pulsed with the indicated peptides (25 μM). E/T=10.

Figure IV-9: Peptide dose-response curve for PUUV G2-specific CD8+ CTL clone 1A-E2. Autologous BLCLs were pulsed with the indicated concentrations of peptide PUUV M_{731-39} and were used as targets in ^{51}Cr-release cytotoxicity assays. E/T=10.
G. Comparison of levels of virus-specific T cell memory with disease severity

An eventual goal of our research is to correlate levels of virus-specific T cell activation with the severity of hantavirus disease. This comparison should provide some insight into the role of T cells in the immunopathology of and/or recovery from hantavirus infection. To evaluate whether there was a correlation between levels of virus-specific T cell memory and severity of the acute illness, we compared total virus-specific memory CD8$^+$ T cell responses (total of precursor frequencies for all epitopes recognized) with various clinical parameters recorded during the acute illness. The clinical characteristics of the donors are shown in Table IV-7, and the total virus-specific memory T cell responses are shown in Table IV-8. Regression analysis demonstrated no statistically significant correlations between the total virus-specific memory T cell response and any clinical measurement, including peak serum creatinine levels, peak urine protein levels, peak hematocrit levels, nadir platelet count, maximum polyuria, blood pressure, duration of fever, length of hospital stay or requirement for dialysis.

We observed weak trends suggesting that the overall level of virus-specific T cell memory may be correlated with maximum urine protein levels, maximum levels of C-reactive protein and duration of fever (Figure IV-10). However, our sample size was extremely small and many more subjects would be needed to identify statistical correlations. Furthermore, since we are only including memory CTL that recognize a maximum of three epitopes (N$_{173-81}$, N$_{204-12}$, N$_{243-51}$) in our calculation of total virus-specific memory T cell responses, it is possible that these totals are not representative of the overall levels of PUUV-specific T cell memory in these individuals. This analysis also excludes individuals who do not express the restricting HLA alleles identified in this study.
Table IV-7: Clinical characteristics of PUUV-immune donors during HFRS

<table>
<thead>
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<th>Donor</th>
<th>Year of HFRS</th>
<th>Max. creat.</th>
<th>Max. urine prot.</th>
<th>Min. platelets</th>
<th>Max C-react. prot.</th>
</tr>
</thead>
<tbody>
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<td>2230</td>
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<td>980</td>
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<td>207</td>
<td>790</td>
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*Highest serum creatinine during hospital care (μM)*
*Highest daily urine protein level (mg/day)*
*Lowest platelet count observed (x10⁹/L)*
*Highest C-reactive protein observed (mg/L)*

<table>
<thead>
<tr>
<th>Donor</th>
<th>Year of HFRS</th>
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<th>Max. polyuria</th>
<th>Duration of fever</th>
<th>Length of time in hosp.</th>
<th>Dialysis</th>
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<td>1985</td>
<td>0.41</td>
<td>5700</td>
<td>8</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>1987</td>
<td>0.54</td>
<td>3250</td>
<td>5</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>1991</td>
<td>0.61</td>
<td>3500</td>
<td>2</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>1988</td>
<td>0.41</td>
<td>2500</td>
<td>7</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>1988</td>
<td>0.62</td>
<td>6410</td>
<td>9</td>
<td>22</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>1984</td>
<td>0.35</td>
<td>8280</td>
<td>20</td>
<td>18</td>
<td>+</td>
</tr>
</tbody>
</table>

*Highest hematocrit measured during hospital care*
*Highest daily urine volume (ml/day)*
*Duration of fever (days)*
*Length of hospital stay (days)*
Table IV-8: Summary of frequencies of virus-specific memory CD8+ T lymphocytes in PUUV-immune donors

<table>
<thead>
<tr>
<th>Donor</th>
<th>HLA</th>
<th>Avg. freq. IFN-γ+ T cells/10⁶ PBMC</th>
<th>Total freq. per 10⁶ PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1, 2</td>
<td>7, 8</td>
<td>304.2</td>
</tr>
<tr>
<td>2</td>
<td>2, 28</td>
<td>8, 44</td>
<td>ND*</td>
</tr>
<tr>
<td>3</td>
<td>3, 24</td>
<td>15, 35</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>1, 3</td>
<td>8, 35</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>1, 24</td>
<td>8, 35</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>1, 3</td>
<td>7, 8</td>
<td>52.2</td>
</tr>
<tr>
<td>7</td>
<td>1, 25</td>
<td>8, 62</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>11, 28</td>
<td>8, 62</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>1, 3</td>
<td>8</td>
<td>ND</td>
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<td>1, 28</td>
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<td>1, 24</td>
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<tr>
<td>12</td>
<td>1, 3</td>
<td>8, 35</td>
<td>ND</td>
</tr>
<tr>
<td>13</td>
<td>1, 2</td>
<td>8</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Not detectable
Figure IV-10: Correlations of virus-specific memory T cell frequency and clinical parameters of disease severity. Total frequencies of virus-specific memory CD8+ T cells were calculated as the sum of average frequencies for 3 epitopes (N_{173-81}, N_{204-12}, N_{243-51}). Total T cell frequencies were plotted against the following clinical parameters: A) Duration of fever (days) B) Maximum urine protein (mg/day) C) Maximum C-reactive protein (mg/L). R^2 values are shown. P values were 0.174 (A), 0.134 (B), and 0.182 (C).
CHAPTER V
DISCUSSION

This thesis provides the first qualitative and quantitative analysis of the memory T cell responses to hantaviruses that cause HFRS. We have shown evidence for memory CTL responses to hantaviruses in individuals following subclinical, laboratory-acquired infections with HTNV and following clinical, naturally-acquired infections with PUUV. We have demonstrated virus-specific CTL activity in bulk T cell cultures and have isolated and characterized virus-specific T cell lines and clones. In the course of these studies, we identified 8 CD8⁺ CTL epitopes (Table V-1) and one CD4⁺ CTL epitope on the N protein of HTNV and PUUV as well as one CD8⁺ CTL epitope on the G2 protein of PUUV. This is the first demonstration of CTL responses to Old World hantaviruses in humans and the first identification of CTL epitopes on these viruses.

We have also developed a quantitative RT-PCR assay to measure the viral RNA copy number during acute PUUV infections. This assay is similar to a quantitative RT-PCR assay designed to measure SNV S segment RNA copy number in patient plasma that was developed previously in our laboratory. This assay will provide information on the quantity of virus circulating in the blood during acute illness, and will allow us to identify potential correlations between virus load and severity of illness caused by PUUV. The combined analysis of the kinetics and magnitude of the viral load and of T cell responses during acute infection will provide insight into the mechanisms of disease pathogenesis.
A. CD8+ CTL RESPONSES TO HTNV AND PUUV

Our aim in these studies was to analyze the specificity and diversity of bulk culture and clonal CTL responses to the Old World hantaviruses HTNV and PUUV.

1. CD8+ CTL responses following sub-clinical infections with HTNV

We first studied CTL responses in three donors who had laboratory-acquired infections with HTNV 5-15 years prior to this study. None of these donors became ill, and HTNV infections were confirmed by identification of HTNV-specific immunoglobulin in the donors' serum. Following in vitro stimulation of PBMC with inactivated virus, we detected hantavirus-specific CTL responses in all 3 donors, indicating that sub-clinical infections with HTNV induced virus-specific CTL responses. The bulk culture CTL response of each donor was directed against a single viral protein: the HTNV N protein in donors A and C, and the HTNV G1 protein in donor B (Figure III-1). The mechanism governing these differences in protein recognition is not known, but may be due to differences in the number of potential CTL epitopes in the proteins that are able to bind particular HLA alleles for presentation to virus-specific CTL.

Isolation of CTL lines from two donors (A & C) lead to the identification of two CD8+ CTL epitopes on the HTNV N protein. Both epitopes were located at the termini of the N protein; one spanned amino acids 12-20 and the other amino acids 421-429 (Figure V-1 and Table V-2). The detection of CD8+ CTL responses in these cultures following stimulation with inactivated virus indicated that the viral peptides were able to access the HLA class I presentation pathway following endocytosis of the inactivated virus. The ability of exogenous antigens to prime the MHC class I presentation pathway, traditionally thought to present only peptides derived from endogenously synthesized proteins, has been described in many systems (191). In the early 1990s, two groups demonstrated that murine splenocytes exposed
to exogenous chicken ovalbumin (OVA) in vitro were able to induce OVA-specific CD8$^+$ T cell responses either in vivo (192) or in vitro (193), illustrating that the exogenous antigen was able to access the MHC class I presentation pathway. Priming of CD8$^+$ CTL by inactivated viruses or soluble viral protein has also been demonstrated for other viruses including influenza, Sendai, and Epstein Barr virus (EBV) (194-196).

Attempts to isolate additional clones from both donors with different specificities were unsuccessful. Additional CTL lines that were isolated all recognized the HTNV N$_{12-20}$ or the N$_{421-429}$ epitope. These data suggested that there were only a limited number of CD8$^+$ CTL epitopes on HTNV N proteins in these individuals. Alternatively, it is possible that the method used to stimulate the PBMC, which presumably involved priming of the exogenous class I pathway, led to the preferential activation and expansion of CTL specific for the epitopes described. Both epitopes are located at the terminal ends of the protein, potentially allowing them to be more readily degraded in endosomes and presented on MHC class I molecules than more internal peptides. However, the mechanisms involved in CD8$^+$ T cell priming by inactivated HTNV were not directly investigated in these studies.

Additional CTL epitopes probably would have been identified if we had stimulated the PBMC with infectious virus. Stimulation of PBMC with live virus would be more likely to result in more efficient loading of antigenic virus epitopes into the classical HLA class I pathway, and thus increase the likelihood of identifying virus-specific CD8$^+$ CTL clones. Schirmbeck et al demonstrated that some, but not all, MHC class I-epitopes are generated during both endogenous and exogenous processing of the hepatitis B virus surface antigen (HBsAg) (197). That study further demonstrated that there are some antigenic epitopes that are only revealed during exogenous processing of viral proteins and subsequent presentation via the class I pathway.
Additional CD8+ T cell epitopes on HTNV may also be identified by screening virus-stimulated bulk cultures against the entire panel of nucleocapsid peptides, or by stimulating PBMC with individual or pooled peptides in ELISPOT assays. The potential of the latter technique was demonstrated in a recent study in which PBMC from EBV+ donors were stimulated with pools of overlapping peptides from the viral EBNA1 protein (196). This approach resulted in the identification of three novel CD8+ CTL epitopes on EBNA1. These epitopes had not been identified previously because traditional approaches for detecting EBV-specific CTL responses relied on stimulation of PBMC with EBV-transformed B cells, in which the presentation of antigenic EBNA1 determinants is prevented due to a domain in EBNA1 that inhibits proteosomal processing, a key step in the endogenous MHC class I processing pathway. This study highlights the importance of the method used to stimulate virus-specific CD8+ T cell responses, and demonstrate the potential of using multiple approaches to identify novel responses. We did not perform these analyses due to the limited availability of PBMC from these donors, and further studies will be required to determine whether the two epitopes described constitute dominant CD8+ T cell epitopes on the HTNV N protein or whether the method of stimulation used resulted in preferential expansion of a small subset of virus-specific T cells.

2. CD8+ CTL responses following HFRS caused by PUUV infection

Our initial studies focused on human CTL responses in individuals who were infected with a laboratory strain of HTNV and who had no clinical disease as a result of the infection. However, many hantavirus infections result in the development of moderate or severe disease, with ample evidence of T cell activation. Analysis of CTL responses after natural infections is important in order to appreciate the magnitude and diversity of CTL responses that are elicited by these viruses during disease. In order to address this issue, we analyzed virus-
specific memory CTL responses in a group of Finnish individuals who had HFRS caused by PUUV infection 6-15 years prior to this study.

Following in vitro stimulation of PBMC with infectious PUUV, we detected CTL activity against one or more PUUV protein in 7/11 donors. The PUUV N and G1 proteins were recognized by most of the donors (6/7 and 5/7, respectively), while the G2 protein was recognized in only 2/7 donors (Table V-1). Greater than one HTNV protein was recognized by bulk culture T cells in 4/7 responding donors. These results demonstrate that multispecific CTL responses can be generated following natural infections with PUUV. This is in contrast to our earlier observation from HTNV-immune donors, in which individual bulk cultures contained detectable CTL activity against only a single HTNV protein. These experiments were performed using infectious virus to stimulate the PBMC, which may have led to more efficient presentation of viral peptides on class I MHC molecules and more efficient CD8+ T cell activation as discussed.

Six CD8+ CTL epitopes on the PUUV N protein were identified from two donors in this study (Table V-2 & Table IV-2). These epitopes were clustered in the center of the N protein, between amino acids 173 and 251, with many of the epitopes overlapping one another. This clustering of CTL epitopes suggests that this may be a highly immunogenic region of the PUUV N protein. It is unclear why so many CD8+ T cell epitopes are clustered at the center of the PUUV N protein. It is possible that this region of the protein is rendered particularly accessible to the proteosomal processing machinery, or that this region contains sequences that are preferred for binding to and/or cleavage by the proteosomal subunits.

The isolation of CD8+ CTL lines specific for multiple epitopes on the PUUV N protein illustrated that the virus-specific CTL response was simultaneously targeted against at least four epitopes in both individuals (Table IV-2). These data demonstrate that there are multiple human CD8+ CTL epitopes on the N protein of PUUV, and that the CD8+ CTL
response in a single individual may be targeted against many epitopes simultaneously. These data also suggest that no one CTL epitope on the PUUV N protein is immunodominant to the exclusion of other epitopes. Again, this observation is distinct from our limited findings from HTNV-immune individuals in which we detected CTL specific for only 2 epitopes on the HTNV N protein in two different donors. As mentioned, infection in the HTNV-immune individuals was sub-clinical. Although levels of viremia were not measured in any of the individuals studied, it is possible that the virus was controlled more efficiently in these individuals compared to those who experienced disease. Rapid elimination of virus may have limited the stimulation of virus-specific cytolytic T lymphocytes and lead to a more restricted CTL response. Studies of memory CTL responses in HTNV-immune individuals who had HFRS following natural infections would be required to determine whether the CTL response to the HTNV N protein is broader than during sub-clinical infection. Conversely, analysis of memory CD8+ CTL responses following sub-clinical infections with PUUV might reveal a more limited virus-specific CTL repertoire than seen after clinical infection.
Table V-1: Summary of bulk culture responses in PBMC from PUUV-immune individuals

<table>
<thead>
<tr>
<th>Donor</th>
<th>PUUV proteins recognized by bulk culture CTL</th>
<th>Specificity of CTL clones isolated (# of clones)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N, G1</td>
<td>N (42), G1 (1)</td>
</tr>
<tr>
<td>2</td>
<td>N</td>
<td>N (19), G2 (1)</td>
</tr>
<tr>
<td>3</td>
<td>N, G1, G2</td>
<td>G2 (1)</td>
</tr>
<tr>
<td>4</td>
<td>N</td>
<td>nd</td>
</tr>
<tr>
<td>5</td>
<td>N, G1</td>
<td>nd</td>
</tr>
<tr>
<td>6</td>
<td>N, G1, G2</td>
<td>nd</td>
</tr>
<tr>
<td>7</td>
<td>G1</td>
<td>nd</td>
</tr>
</tbody>
</table>
Table V-2: Summary of CD8+ CTL epitopes identified on HTNV, PUUV and SNV.

<table>
<thead>
<tr>
<th>Virus</th>
<th>CTL Epitope</th>
<th>Restricting HLA</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTNV</td>
<td>N_{12-20} *</td>
<td>B51</td>
<td>NAHEGQLVI</td>
</tr>
<tr>
<td>HTNV</td>
<td>N_{421-429} *</td>
<td>A1</td>
<td>ISNQEPLKL</td>
</tr>
<tr>
<td>PUUV</td>
<td>N_{173-81} *</td>
<td>B7,B8</td>
<td>RPKHYVSM</td>
</tr>
<tr>
<td>PUUV</td>
<td>N_{204-12} *</td>
<td>A2</td>
<td>GLFPTQIQV</td>
</tr>
<tr>
<td>PUUV</td>
<td>N_{212-20} *</td>
<td>?</td>
<td>VRNIMSPVM</td>
</tr>
<tr>
<td>PUUV</td>
<td>N_{243-51} *</td>
<td>B8</td>
<td>ECPFIKPEV</td>
</tr>
<tr>
<td>PUUV</td>
<td>N_{164-78} *</td>
<td>A28</td>
<td>TSFEDINGIRRPKHL</td>
</tr>
<tr>
<td>PUUV</td>
<td>N_{236-50} *</td>
<td>?</td>
<td>IREFMEKECPFIKPE</td>
</tr>
<tr>
<td>PUUV</td>
<td>M_{731-39} (G2) *</td>
<td>A24</td>
<td>HWMDATFNL</td>
</tr>
<tr>
<td>SNV</td>
<td>N_{151-39} 1</td>
<td>B35.01</td>
<td>LPIILKALY</td>
</tr>
<tr>
<td>SNV</td>
<td>N_{234-42} 1</td>
<td>Cw7</td>
<td>ERIDDFLAA</td>
</tr>
<tr>
<td>SNV</td>
<td>M_{664-673} (G2) 2</td>
<td>B35.01</td>
<td>TAHGVGIIPM</td>
</tr>
<tr>
<td>SNV</td>
<td>M_{746-755} (G2) 2</td>
<td>B35.01</td>
<td>YPWQTAKCFF</td>
</tr>
</tbody>
</table>

*Identified in this thesis

1 (52)

2 unpublished data
3. **HLA restriction of hantavirus-specific CTL**

Genetic factors appear to play an important role in the pathology of hantavirus disease. As discussed, the HLA haplotype B8, DR3 was shown to be associated with more severe HFRS caused by PUUV infection. The PUUV N-specific CD8+ CTL lines that we isolated from two immune individuals were restricted by a variety of HLA alleles, including A2, A28, B7 or B8, indicating that many different HLA alleles are capable of presenting antigenic peptides from the PUUV N protein. The identification of CTL epitopes on the PUUV N protein will be of great value for future studies of acute T cell responses during HFRS. This information will allow the generation of reagents, such as HLA/peptide tetramers, that can be used to track the virus-specific CD8+ T cell response during the acute phase of disease. These studies will provide insight into the kinetics and magnitude of CD8+ T cell responses during acute HFRS.

We were particularly interested in the CD8+ T cell epitopes that were restricted by HLA B8, since this HLA allele is associated with severe disease, and is thus of interest for studies of acute HFRS caused by PUUV. Two HLA B8-restricted epitopes were identified: N\textsubscript{173-81} and N\textsubscript{243-51}, and memory CD8+ T cells specific for these epitopes could be detected in the PBMC from 2/12 and 8/12 immune individuals, respectively (Table IV-3). The average frequencies of memory CD8+ T cells specific for the N\textsubscript{173-81} epitope (52-304/10\textsuperscript{6} PBMC) were slightly higher than those specific for the N\textsubscript{243-51} epitope (13-188/10\textsuperscript{6} PBMC), but these cells were detectable in only 17% (2/12) of the immune donors tested. In contrast, memory CD8+ T cells specific for the N\textsubscript{243-51} epitope were detectable in the majority (67%) of immune donors tested, making this epitope an attractive candidate for analysis of acute T cell responses during HFRS. Memory CD8+ CTL specific for the HLA A2 epitope (N\textsubscript{204-12}) were detectable in 100% (3/3) of immune donors analyzed. HLA A2 is a common allele in Scandinavian populations, being present in approximately 40% of the population (198).
high frequency of this HLA allele in Scandinavian populations, combined with the likelihood that many infected individuals will possess CD8^+ T cells specific for this epitope, makes it another attractive candidate for studies of acute T cell responses. Analysis of this epitope will also provide an essential comparison for the quantitation of HLA B8-restricted T cells during these studies.

Interestingly, none of the donors had detectable numbers of memory CD8^+ T cells specific for the A28-restricted epitope N_{164-78}, or for the A24-restricted epitope on the G2 protein (M_{731-39}), including the donors from whose PBMC the specific CTL lines were isolated (Table IV-3). This suggests that these epitopes may be subdominant compared to the A2- and B8-restricted epitopes.

SNV-specific CTL lines that recognized determinants on the SNV N and G2 proteins were shown to be predominantly B35-restricted (Table V-2) (115) (unpublished observations). This is interesting since the HLA allele B35 has been shown to be associated with severe HPS caused by SNV, and it suggests that B35-restricted CTL responses may be dominant in these donors during acute HPS.

4. Magnitude and heterogeneity of memory CD8^+ CTL in PUUV-immune individuals

ELISPOT data presented in Chapter IV demonstrates that CTL specific for the PUUV N protein are abundant in the memory T cell pool of these donors, especially when one considers the duration of time since exposure to the virus (6-15 years) and the unlikelihood of re-infection. CTL specific for N_{204-12} were found at precursor frequencies ranging from 35-217/10^6 PBMC, and were especially abundant in donor 13. CTL specific for N_{173-81} were detected at precursor frequencies reaching 300/10^6 PBMC in donor 1 (Figure IV-5, Table V-3). Finally, CTLs specific for N_{243-51} were detected at frequencies ranging from 36-188/10^6 PBMC, and were most abundant in donors 11 and 13 (Figure IV-5A, Table V-3).
Table V-3: Frequencies of PUUV N-specific memory CD8⁺ T cells in immune donors

<table>
<thead>
<tr>
<th>Donor</th>
<th>Years since acute infect.</th>
<th>Avg. freq. IFN-γ⁺ T cells/10⁶ PBMC</th>
<th>Total freq./10⁶ PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N₁₇₃-₈₂(B7.8)</td>
<td>N₂₀₄-₁₂ (A2)</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>304.2</td>
<td>87.2</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>ND*</td>
<td>49.8</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>52.2</td>
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</tr>
<tr>
<td>7</td>
<td>12</td>
<td>ND</td>
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</tr>
<tr>
<td>8</td>
<td>15</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>13</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>12</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>16</td>
<td>ND</td>
<td>200.4</td>
</tr>
</tbody>
</table>
These frequencies of PUUV-specific memory CTL are considerably higher than many frequencies of CTL specific for commonly recognized epitopes on influenza A virus as measured by INF-γ ELISPOT assays (187, 190). In one study, the highest precursor frequencies of memory CTL specific for the flu epitopes M1_{58-66} (A2-restricted) and NP_{380-88} (B8-restricted) ranged from 1/3,500-1/111,000 (9-286/10^6 PBMC) and 1/15,000-1/67,000 (15-67/10^6 PBMC), respectively (187). Like PUUV, influenza virus causes an acute infection that is effectively cleared by the immune system. One major difference between these two viruses is that influenza virus can periodically boost virus-specific CTL responses by re-infecting individuals with existing influenza virus-specific T cell memory. This does not appear to happen in the hantavirus system, in which secondary infections have never been reported. High levels of PUUV-specific antibodies circulate following resolution of infection and these antibodies can be detected for many years (80, 83). High antibody titers, in combination with the relatively low mutation rate of the PUUV envelope glycoproteins, probably prevent the virus from establishing a second infection in an immune individual. Considering the unlikelihood that the PUUV-specific CTL response in these donors had been boosted since the primary infection, the frequencies of memory CTL observed in these donors were very high.

Measles virus infection provides an apt comparison to hantavirus infection, since measles virus immunity also appears to be lifelong. Frequency analysis of measles virus-specific CTL in healthy adult donors who had childhood infections with measles reported that 100-960 cells per 10^6 PBMC were virus-specific (199). This group used measles-infected BLCLs, rather than defined peptide epitopes, as stimulator cells in IFN-γ ELISPOT assays. These numbers therefore reflect the total of all measles virus-specific T cells capable of producing IFN-γ in these donors. The total frequency of the PUUV-specific T cells analyzed
in the present study was between 15 and 431/10^6 PBMC (Table V-3). Since we measured the responses to only 4 CD8+ CTL epitopes, restricted by only 4 possible HLA alleles (A2, A28, B7,B8), we are likely to be underestimating the total PUUV-specific T cells responses possible in the context of a given individual’s HLA alleles. In addition, studies in which virus-specific T cell frequencies were measured by various techniques demonstrate that the ELISPOT assay may be up to 10-fold less sensitive than intracellular IFN-γ staining and tetramer staining (200, 201). Thus, our use of the IFN-γ ELISPOT assay may also result in an underestimation of the frequency of PUUV N-specific CD8+ T cells. The frequencies of virus-specific CTL responses measured in the present study are also comparable to the frequencies of many virus-specific CTL in adult donors with chronic virus infections such as EBV and HIV (201-204). In these systems, CTL precursor frequencies are often very high, probably due to periodic reactivation as a result of the persisting virus infection.

It is likely that the memory CD8+ T cells that are abundant in the memory T cell pool of these individuals were present during the acute infection. Several studies have shown that CTL that are present at high frequency during acute infection are also detectable at lower levels in the memory repertoire, and that hierarchies of epitope recognition seen during acute infection are largely maintained in memory (205, 206). Studies carried out during acute PUUV infections will be required to assess the relative levels of epitope-specific CTL present during the acute response and determine whether they mirror the hierarchies of epitope-specific responses in the memory population. We predict that the PUUV epitopes defined in this study will be detectable at high levels in individuals with acute HFRS.

This study provides evidence that PUUV-specific memory CTL are not only abundant in the memory T cell pool, but are also phenotypically heterogeneous. TCR Vβ analysis of 9 CTL lines specific for PUUV N204-12 revealed that at least 5 different Vβ chains are
represented in this CTL population (Table IV-6). Several recent studies have examined the TCR diversity of clones specific for a single, often immunodominant, CTL. The results of these studies have been quite variable. In some systems, epitope specific CTL displayed restricted TCR repertoires in which one or a few clones dominated and a bias in Vβ chain usage was seen (207-209). Other studies demonstrated extremely diverse TCR repertoires within populations of CTL with the same specificity (210, 211). The majority of these studies have been carried out in viral systems in which the virus establishes a persistent infection, such as HIV, HTLV and EBV (208, 209). Fewer studies have involved acute viral infections, in which the virus is successfully cleared by the immune system; the most detailed of these are studies of influenza virus. The TCR diversity seen here is in contrast to the observed bias for Vβ17 expression in CTL specific for the influenza M157-68 epitope (207, 212). The implications of this heterogeneity are unknown, but the existence of many T cell clones with the same specificity may contribute to a higher level of T cell expansion during the acute infection.

Although this was not specifically addressed in this study, the TCR diversity of the CTL specific for N204-12 may result from the immunodominant nature of this epitope. A study of CTL responses to L. monocytogenes in mice demonstrated that CTL lines specific for dominant H-2K^d-restricted epitopes express diverse TCR Vβ chains, while CTL specific for subdominant H-2K^d-restricted epitopes express a more restricted range of Vβ chains (213). In that study, spleen cells from L. monocytogenes-immunized mice were stimulated in vitro with dominant or subdominant peptides at optimal concentrations. Following peptide-specific expansion, >90% of the T cells in culture were peptide specific as determined by H2-K^d tetramer staining. Remarkably, when these CTL lines were stained with mAbs specific for 13 different Vβ TCR chains, the cell lines specific for the immunodominant epitopes LLO 91-99
& p60 217-225 showed an extremely diverse TCR-Vβ repertoire. In contrast, the cell lines specific for the subdominant epitopes p60 449-457 and mpl 84-92 had very restricted TCR-Vβ usage, with only one TCR Vβ chain (Vβ14) being expressed by the CTL line specific for mpl 84-92.

The different peptide concentrations required by these CTL for target cell recognition suggests that these TCR also have a wide range of binding affinities for the peptide/HLA complex (Figure IV-3A). This observation emphasizes the diversity of the CTL population that recognizes the N204-12 epitope. Distinct patterns of cross-reactivity among the CTL lines specific for several other PUUV epitopes also suggest that these populations are polyclonal (Table IV-4).

5. Immunodominance of hantavirus N protein as a CTL target

The majority of the HTNV- and PUUV-immune individuals with detectable hantavirus-specific CTL in bulk culture responded to the N protein. Two of three of the HTNV-immune donors had detectable CTL specific for the HTNV N protein in bulk culture, and all of the virus-specific CTL lines isolated from these donors were HTNV N-specific. In addition, bulk cultures from 6 of the 7 PUUV-immune subjects contained PUUV N-specific CTL, and the vast majority of CTL lines isolated from two donors were N-specific (61/63). Further, we demonstrated that memory CD8+ T cells specific for one or more epitopes on the PUUV N protein were detectable in 8 PUUV immune donors, even more than a decade after the acute infection. In contrast, memory CD8+ T cells specific for an epitope on the PUUV G2 protein (M73-39) were not detectable in any of the HLA A24+ donors tested.

The relative abundance of CTL lines specific for N protein compared to those specific for the G1 and G2 glycoproteins, the fact that the majority of hantavirus-immune donors have
detectable N-specific CTL responses in bulk culture, and the high frequency of PUUV N-specific memory CTL in immune donors, suggest that the N protein may be the dominant target of the human CTL response during hantavirus infections. Many factors could contribute to the dominance of N-specific CD8+ T cell responses. Properties of the PUUV N protein itself may influence the generation of antigenic peptides from the protein, and contribute to immunodominance. For example, the rate and temporal sequence of translation of the viral gene products is proportional to the rate of peptide generation, and this could influence the rate of processing and presentation of viral peptides (214). The hantavirus N protein is expressed early in infected cells, and may represent the most abundant viral gene product. In SNV infected Vero E6 cells, Hutchinson and colleagues demonstrated that the N mRNA appeared earlier and accumulated to higher levels than the glycoprotein or polymerase mRNA species (215). Another study demonstrated that the kinetics of the expression and accumulation of the PUUV N protein precisely mirrored the kinetics of SNV N mRNA expression, with protein detectable by 4 hours post-infection and significant accumulation evident 48 hours post-infection (106). However, in that study, the expression of the N protein was not compared to the other PUUV gene products. The studies described here suggest that the N protein is a dominant target of the human anti-viral T cell response, although the mechanisms that may contribute to this dominance have not been investigated.

We were able to isolate three PUUV glycoproteins-specific CTL lines: 1 G1-specific CTL lines from donor 1, 1G2-specific CTL line from donor 2, and 1 G2-specific CTL line from donor 3. This demonstrates that there are antigenic CTL determinants on these proteins. The paucity of CTL lines specific for G1 and G2 may reflect the presence of fewer antigenic epitopes on the envelope glycoproteins relative to the N protein, or simply that glycoprotein-specific precursor CTL are less abundant than those specific for the N protein. Alternatively, since the expression of the glycoproteins in the recombinant vaccinia viruses used to infect
target cells for $^{51}$Cr-release assays was very low relative to that of the N protein (M.T. Terajima, manuscript in preparation), there may be insufficient numbers of peptide-MHC complexes on the surface of the target cells to allow sensitization of glycoproteins-specific CTL clones. Other studies have demonstrated that the abundance of peptide-MHC complexes on the surface of a cell can vary considerably, depending on the system used to express the protein from which the antigenic peptide is derived. Using HPLC extraction of peptides bound to class I MHC molecules in mice, Rammensee et al. demonstrated that a Db-restricted influenza nucleoprotein (NP) epitope was present at approximately 300 copies per influenza virus infected cell (216). Using the same method, a later study showed that only 30 copies/cell of the same epitope were present when a recombinant vaccinia virus expressing the NP protein was used to infect the cells (217). It is thus possible that our inability to isolate more PUUV glycoprotein-specific CTL lines simply reflects the inefficiency of the expression of these proteins in vaccinia virus vectors, although this was not specifically addressed.

Previous studies of virus-specific CTL responses during acute BPS caused by SNV infection resulted in the identification of 2 CD8$^+$ CTL epitopes on the SNV N protein, and 2 CD8$^+$ epitopes on the SNV G2 protein (Table V-2). This demonstrates the existence of CD8$^+$ T cell epitopes on multiple viral proteins, but does not suggest that one protein elicits a dominant response. Studies performed during acute HPS comparing the relative magnitudes of the CD8$^+$ T cell response to the epitopes on N and G2 may reveal whether or not the N protein is also the dominant target of SNV-specific CTL.

**B. CROSS-REACTIVITY OF HANTAVIRUS-SPECIFIC CTL LINES**

There are at least 20 distinct hantaviruses, approximately half of which are known to cause human disease. We are interested in whether protective immunity against one
hantavirus might confer immunity against other hantaviruses. There have been no reported cases of hantavirus re-infection in individuals with existing immunity to a particular hantavirus, and it is thus thought that recovery from hantaviral infection results in life-long immunity. However, it is not known whether immunity to one virus may confer some protection against other strains of hantavirus or, alternatively, whether immunity against one hantavirus may predispose an individual to immunopathology in the event of a second infection. Although many different hantaviruses are thought to be very geographically isolated from one another, recent studies have shown that there are regions in which more than one distinct hantavirus co-circulate (188). The co-circulation of different hantaviruses, as well as the possibility of exposure to different viruses during travel, highlights the potential importance of cross-reactive immunity.

1. Cross-reactivity of CTL lines isolated from HTNV-immune donors

Cross-reactivity studies conducted with the CTL lines isolated from the HTNV-immune donors revealed that some CTL were cross-reactive with many different hantaviruses, while others were highly specific. Most of the observed cross-reactivity (or lack thereof) could be attributed to sequence homology among the different viruses at the given epitope. However, among CTL lines that recognized the same epitope, complex patterns of cross-reactivity were noted.

The three CTL lines specific for amino acids 12-20 (NAHEGQLVI) were all restricted by HLA B51 and were either HTNV-specific or recognized only the very similar SEOV epitope (SAHEGQLVI) (Figure III-4, III-6A). The consensus motif for peptides binding to HLA B51 consists of anchor residues at positions 2 (A,P,G) and 9 (L,V,I) (189). This HTNV N peptide fits the consensus B51 peptide binding motif with an alanine (A) at position 2 and isoleucine (I) at position 9. It is not surprising that the clones specific for this epitope failed to
recognize the corresponding region of other hantaviruses since this region of the nucleocapsid protein is quite variable among different viruses, with many of the amino acid differences occurring at the anchor positions for HLA binding (Fig. III-6A). The corresponding epitope from DOBV differs from the HTNV epitope by only one amino acid. However, the amino acid change in the DOBV epitope is located at the anchor position 2 (A13→N). We hypothesized that this substitution may result in an inability of the peptide to bind the HLA B51 molecule and this could explain the lack of recognition by the CTL lines. It is interesting to note that the HLA B51 is very common among populations in Asia, where HTNV is endemic, being present in a higher portion of the population (8-12%) than most other HLA alleles (198).

The CTL lines that recognize the C-terminal nine amino acids of the HTNV N protein (421-429: ISNQEPLKL) are restricted by HLA A1 and are cross-reactive against several different hantaviral sequences (Figure V-4, III-6B). Interestingly, this peptide does not fit the consensus HLA A1 binding motif in which an acidic residue (D, E) is preferred at position three and a tyrosine (Y) is highly preferred at the anchor position 9 (189, 218). The restricting allele for this CTL epitope, HLA-A1, is common in North American Caucasian populations (17-19%) but is relatively rare among Asian populations (0.5-5%) (198), so the importance of this epitope among individuals in areas where HTNV is endemic remains to be determined. Studies of CTL responses in HFRS patients from Asian countries will be required to determine whether this epitope is a target of CTL during natural infections.

The N_{421-429} epitope is identical among various hantaviruses including SNV, ANDV, NYV, and DOBV and differs from the PUUV, PHV and BAYV epitope by a single conservative change at position 9 (L429→I). The corresponding epitope from SEOV also differs by one amino acid with a substitution at position 7 (L427→M) (Figure III-6B).
sequence conservation at this epitope and the recognition of target cells expressing SNV N protein by CTL, suggest that infection with HTNV induces some cross-reactive T cell responses. This cross-reactive epitope is particularly interesting in that it is identical among not only closely related viruses that cause HFRS (e.g. HTNV and DOBV) but also among viruses that are more distantly related and cause distinct syndromes (e.g. HTNV and SNV).

The data presented here demonstrate that infection with HTNV results in the development of both HTNV-specific and hantavirus cross-reactive CD8+ T cell responses, with distinct patterns of cross-reactivity among CTL lines that recognize the same epitope (Table V-4). This suggests that immune individuals may be protected to some extent from secondary infections with other hantaviruses. Additionally, individuals vaccinated with a vaccinia virus encoding the HTNV M segment had detectable proliferative responses to both HTNV and SNV, indicating that a cross-reactive CD4+ T cell response was generated in these individuals (175). The SNV-specific CTL lines established in our laboratory also included both SNV-specific and cross-reactive lines (115).

Cross-reactivity of virus-specific CTL is a common phenomenon that has been described in many viral systems. Individuals who received a live, attenuated dengue virus immunization develop both serotype-specific and serotype cross-reactive T cell responses (117, 219, 220). Similarly, studies of human CTL responses to influenza viruses demonstrated that CTL specific for epitopes on conserved, internal proteins can recognize other strains of influenza virus that infect humans as well as those that target avian and swine species (190, 221). The ability of human memory CTL responses to recognize determinants from avian influenza viruses was speculated to account for the limited spread of the “Hong Kong” influenza virus from chickens to humans in 1997.

Murine studies have demonstrated that immunization with one type of hantavirus elicits protective CTL responses that are cross-reactive with other hantaviruses (93, 222). In
those studies, spleen cells from PUUV or PHV-immunized mice were shown to have cytolytic activity against HTNV-infected target cells in $^{51}$Cr-release assays, following short term in vitro stimulation with HTNV-infected macrophages (222). Additionally, the transfer of PUUV-immune splenocytes in nude mice was shown to significantly reduce titers of HTNV in the lung and spleen when transferred 1 day prior to challenge with HTNV. This demonstrates that hantavirus-specific T cells were able to confer cross-reactive protection against heterologous viruses in mice. A similar cross-reactive response to SEOV was induced following immunization of mice with HTNV (93).

This is the first demonstration that cross-reactive CD$^8^+$ CTL responses are also generated in humans following infection with HTNV. In addition to providing protection against subsequent infection, it is also possible that activation of cross-reactive memory CD$^4^+$ and CD$^8^+$ T cells may be deleterious in the event of re-infection, by contributing to excessive activation of the immune response and resulting immunopathology.
Table V-4: Cross-reactivity of CD8+ CTL lines isolated from HTNV-immune subjects

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Donor</th>
<th>CTL Line</th>
<th>HTNV</th>
<th>SNV</th>
<th>PUUV</th>
<th>SEOV</th>
<th>DOBV</th>
<th>ANDV</th>
<th>PHV</th>
</tr>
</thead>
<tbody>
<tr>
<td>N12-20</td>
<td>A</td>
<td>3-G7</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>10-E2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3A-G3</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N421-429</td>
<td>A</td>
<td>1-C8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1A-B7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3A-B8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3A-C4</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3A-C10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
2. Cross-reactivity of CTL lines isolated from PUUV-immune donors

The epitopes identified in this study are clustered near the center of the PUUV N protein in a region that shares relatively little sequence identity among the different hantaviruses. It was not surprising, therefore, to find that the majority of the CTL lines isolated in this study failed to recognize target cells expressing the N protein from either HTNV or SNV (Tables IV-4 & IV-5). However, we did find three CTL lines (10-F5, 1B-G9 and 3-B8) that displayed cross-reactive lysis of target cells infected with recombinant vaccinia viruses expressing either the HTNV or SNV N protein. The epitope recognized by 10-F5 (N\textsubscript{173-81}) is well conserved between PUUV, HTNV and SNV (Table IV-5). CTL line 10-F5 recognizes the epitope from PUUV and HTNV, which differ by only two amino acids. However, CTL line 3-C8, which is specific for the same peptide/HLA combination, did not recognize the HTNV N protein. Similarly, CTL line 1B-G9 was the only one of seven lines specific for N\textsubscript{236-50} that recognized targets expressing both PUUV N and SNV N (Table IV-4). In this case, there was little sequence conservation at this epitope among the viruses. Finally, the CTL line specific for PUUV N\textsubscript{164-78} (3-B8) cross-reacted with targets expressing the HTNV, but not the SNV, N protein. The minimal epitope recognized by this cell line remains to be characterized, but the 15-mer sequence differs between PUUV and HTNV by only 3 amino acids, all of which are conservative substitutions (T→S, I→V, R→K) that are likely to preserve the integrity of the antigenic epitope. The corresponding SNV sequence differs from the PUUV sequence by 6 amino acids, all of which constitute conservative substitutions.

In contrast to our earlier data, which suggested that hantavirus infections elicit both virus-specific and cross-reactive CTL responses, these data suggest that the virus-specific T cell responses in these individuals were largely PUUV-specific (Table V-5). Additional studies of acute and memory CTL responses in infected or immune individuals are required to
determine the extent of cross-reactivity of cytotoxic T cells that are generated during hantavirus infections.

Table V-5: Cross-reactivity of CD8⁺ CTL lines isolated from PUUV-immune subjects

<table>
<thead>
<tr>
<th>Epitope</th>
<th>HLA Restriction</th>
<th># CTL lines</th>
<th># x-reactive with HTNV</th>
<th># x-reactive with SNV</th>
</tr>
</thead>
<tbody>
<tr>
<td>N204-12</td>
<td>A2</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N173-81</td>
<td>B7</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>N173-81</td>
<td>B8</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N236-50</td>
<td>?</td>
<td>7</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>N212-20</td>
<td>?</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N164-78</td>
<td>A28</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
C. SUMMARY

This thesis provides the first analysis of human memory CD8+ T lymphocyte responses to the Old World hantaviruses HTNV and PUUV. Overall, the data from these studies suggest that the N protein is the dominant target of CD8+ T cell responses during PUUV infection and, although the data are very limited, the N protein may also be immunodominant during infection with HTNV and other hantaviruses. The PUUV N-specific CTL response, which is focused on a cluster of epitopes between amino acids 173 and 215, appears to be broad, heterogeneous and largely PUUV-specific. The data also show that PUUV N-specific CTL persists for more than a decade after the acute infection at high frequencies that are comparable to or exceed those measured for other acute viruses such as influenza and measles, as well as for chronic viruses such as EBV and HIV.

In summary, the results presented in this thesis examine virus-specific memory T lymphocyte responses following sub-clinical infections and clinical, naturally-acquired infections with hantaviruses that cause HFRS. The major findings are:

1. CD4+ and CD8+ virus-specific T cell responses can be detected following sub-clinical, laboratory-acquired infections with HTNV, the prototype hantavirus. Two novel CD8+ CTL epitopes were identified on the HTNV N protein. Sub-clinical infections with HTNV induced both HTNV-specific and hantavirus-cross-reactive CD8+ T cell responses.

2. CD4+ and CD8+ virus-specific T cell responses can be detected in healthy adults following resolution of HFRS caused by PUUV infections, and the PUUV N protein appears to be the dominant target of the virus-specific T cell response in these individuals. Infection elicits a broad response to the PUUV N protein, with CTL specific for multiple epitopes being detectable in a single individual. Six novel CD8+ CTL epitopes and one novel CD4+ CTL
epitope were defined on the PUUV N protein and the majority of T cell lines specific for these epitopes were PUUV-specific.

3. CD8+ memory CTL specific for several epitopes on the PUUV N protein can persist at high frequency in the peripheral blood for at least 15 years after resolution of the acute infection, despite the fact that memory CD8+ T cell responses are unlikely to be boosted during that time.

4. A quantitative RT-PCR assay was developed and optimized and may be used to measure levels of PUUV RNA in acute patient sera. This assay will provide critical information regarding the kinetics and magnitude of the virus load during PUUV infection.

These studies were initiated as a result of our hypothesis that virus-specific T cells may be involved in immunopathogenesis during HFRS. While the studies described in this thesis do not elucidate the role that T cells play in the pathogenesis of disease, they provide the information necessary to address this question. As a direct result of these studies, it will be possible to directly examine PUUV-specific CD8+ T cell responses during acute HFRS. The identification of epitopes on PUUV will allow us to track virus-specific T cell responses through the acute phase of disease and into convalescence using sensitive techniques such as peptide/MHC tetramer staining and intracellular cytokine staining. These studies will provide the first clues regarding the kinetics and magnitude of the CD8+ T cell response during acute HFRS. This quantitative information can be compared to virus load and to clinical parameters that reflect disease severity, and will provide insight into the potential role of CD8+ T lymphocytes in the pathogenesis of HFRS. More generally, this system will provide a well-
defined human model of virus infection in which to study basic immunological concepts such as the establishment and maintenance of long-term virus-specific T cell memory.
APPENDIX I

DESIGN AND OPTIMIZATION OF A QUANTITATIVE RT-PCR ASSAY TO MEASURE PUUV RNA COPY NUMBER IN PLASMA OF PATIENTS WITH HFRS

The pathogenesis of HFRS is not well defined, but we and others hypothesize that T lymphocyte activation and cytokine responses are likely to contribute to disease pathogenesis. Another parameter that may impact the severity of illness resulting from hantavirus infection is the virus burden. Higher virus titers may result in a more robust immune response and thus contribute to immunopathology. To better understand the role of the virus load in the pathogenesis of disease, we developed a quantitative RT-PCR assay to measure PUUV S segment RNA copy number in the plasma of patients with HFRS.

Our laboratory had previously designed a similar RT-PCR assay to measure SNV S segment RNA copy number in patients with HPS (108). In that study, RNA copy number was measured in 26 patients with HPS, at one or more time points during the acute illness. The results showed that viral RNA copy number correlated with several parameters of disease severity. There was a positive correlation between vRNA copy number and peak hematocrit levels, and an inverse correlation with nadir platelet count, suggesting that higher viral burden is associated with more severe illness. In addition, the mean vRNA copy number was $1 \log_{10}$ higher in fatal cases than in survivors, again suggesting that higher virus titers are correlated with greater disease severity. The previous study also showed that, in 8 patients who survived, vRNA copy numbers decreased to undetectable levels in samples taken after fever resolution. We predict that vRNA copy number may also correlate with severity of HFRS following PUUV infection.

A previous study demonstrated that PUUV S segment RNA could be amplified by RT-PCR from the PBMC and urine of patients with HFRS (158). In that study, 3 of 15
PBMC and 4 of 6 urine specimens were positive for PUUV S segment vRNA. Interestingly, 5 of 6 PCR-positive patients (83%) expressed the HLA haplotype B8, DR3, which was previously shown to be correlated to more severe illness following PUUV infection. In contrast, only 1 of 11 (9%) PCR-negative patients possessed this haplotype. The ability to detect vRNA by PCR also correlated with several clinical parameters that reflect disease severity including thrombocytopenia, requirement for dialysis and length of hospital stay, although these correlations were not statistically significant. These data suggest that virus load in PUUV infection may also be associated with more severe disease, as we have seen in HPS caused by SNV.

A. Design of a competitor plasmid for PUUV-specific RT-PCR.

To create a competitor plasmid from which to generate negative stranded RNA for PUUV-specific RT-PCR, we used a plasmid generated previously in our lab (pFIR2+118) (108), which contains the SNV S segment cDNA (nt 42-309) and an additional 118-base pair segment (see Materials and Methods). To use this competitor plasmid for a PUUV-specific PCR, we engineered PUUV-specific primer binding sites at the ends of the SNV S segment by PCR amplification of the SNV S segment. The primers used to amplify the segment were PUU/SNV “hybrid” primers, and PCR amplification resulted in a product identical to the starting product with PUUV-specific primer binding sites at either end of the SNV S segment (Figure V-1). Although the RNA that results from this competitor plasmid will consist mostly of SNV S segment sequence, we hypothesized that the PCR amplification of this RNA would be comparable to the amplification of PUUV S segment RNA, since the nucleotide sequence of the PUUV and SNV S segments are 70% identical in this region and the sequences do not contain highly G/C rich regions. Positive control RNA was generated from a plasmid, pSP72-1tr, that contains a portion of the PUUV K27 S segment.
Figure A-1: Schematic showing the construction of the competitor plasmid for PUU virus-specific RT-PCR
B. Sensitivity of RT-PCR for amplification of positive control and competitor RNA.

To assess the sensitivity of our RT-PCR system, we made serial 10-fold dilutions of the competitor and positive control RNA and added a known quantity of RNA (S segment copy number) to each PCR reaction tube. Figure V-2A shows the sensitivity of the RT-PCR for the competitor RNA. A band is visible in the reaction containing $1 \times 10^3$ RNA copies, and a faint band is visible in the reaction containing $1 \times 10^2$ RNA copies. This indicates that at least 100-1000 competitor RNA copies are required for detection in this assay. The sensitivity of the RT-PCR assay for the positive control RNA was comparable to the sensitivity for the competitor RNA, and required approximately $1 \times 10^3$ copies for detection (Figure V-2B).

To quantitate the viral RNA in patient plasma, the RT-PCR reaction is performed with both the competitor RNA (known concentration) and the viral RNA (unknown concentration) in the same tube. To insure that the RT-PCR assay amplified both the competitor RNA and viral RNA appropriately in the same tube, we placed a constant concentration of positive control RNA in each tube and added serial 10-fold dilutions of competitor RNA to the successive tubes. The band intensities of the resulting PCR products should be equivalent in the tubes containing equivalent numbers of copies of positive control and competitor RNA species. Figure V-2C shows that the band intensities of the competitor and control RNA products are indeed comparable in the reactions that contained equal numbers of copies of the two RNA species. We did not see any non-specific PCR products in any of these experiments.
A.

Competitor: RNA copies/rxn:

\[
\begin{array}{cccccc}
10^6 & 10^5 & 10^4 & 10^3 & 10^2 \\
\end{array}
\]

B.

Positive Control: RNA copies/rxn:

\[
\begin{array}{cccccc}
10^8 & 10^7 & 10^6 & 10^5 & 10^4 & 10^3 & 10^2 \\
\end{array}
\]

C.

Competitor: RNA copies/rxn:

\[
\begin{array}{cccccc}
10^6 & 10^5 & 10^6 & 10^5 & 10^8 & 10^7 & 10^6 & 10^5 & 10^4 & (-) \\
\end{array}
\]

Pos. control: RNA copies/rxn:

\[
\begin{array}{cccc}
10^7 & 10^6 & 10^5 & 10^4 \\
\end{array}
\]

Figure A-2: Sensitivity of quantitative RT-PCR assay for detection of PUUV S segment RNA. 1 μl of competitor RNA (FIR2+118-8) and/or positive control RNA (pSP72-ltr) was amplified in each reaction tube. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.
Since the prior optimization experiments utilized only in vitro transcribed RNA, it was necessary to show that viral RNA was also amplified efficiently under these conditions. We amplified vRNA isolated from supernatants of Vero E6 cells infected with PUUV (strain K27) after the first or second passage. A constant amount of viral RNA (1 μl) and the indicated amount of competitor RNA was added to each tube. Figure V-3 demonstrates that the PUUV vRNA was amplified efficiently, and that the supernatant from the first virus passage contained approximately \(10^5\) RNA copies/μl. The supernatant from the second RNA passage contained between \(10^5\) and \(10^6\) RNA copies/ml. These data demonstrated that PUUV RNA could be amplified efficiently using this newly developed RT-PCR assay.

**Figure A-3**: Amplification of PUUV (K27) virus grown in Vero E6 cells. Supernatants were collected from PUUV after passage 1 or 2 and stored at -70°C until use. vRNA was prepared from 140 μl of infected cell supernatant using the QIAamp viral RNA isolation kit (Qiagen, Valencia, CA) and 1 μl of vRNA was included in each reaction tube. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.
C. Specificity of RT-PCR.

To show that the RT-PCR assay was specific for PUUV RNA and would not amplify viral RNA from other hantaviruses, we isolated viral RNA from several different strains of hantavirus including PUUV, SNV, HTNV, and SEOV. One µl of vRNA was added to each tube and RT-PCR was performed in the absence of competitor RNA. Figure V-4 shows that a PCR product was visible only in the reaction containing the PUUV RNA and the positive control RNA, and no bands were visible in the reactions containing RNA isolated from other hantaviruses. These data indicated that the RT-PCR was specific for PUUV RNA.

![Figure A-4: Specificity of PUUV RT-PCR assay. Viral RNA was isolated from 140 µl of supernatant from passaged viruses including PUUV, SNV, HTNV and SEOV. 1 µl of vRNA or positive control RNA (pSP72-1tr) was amplified in each reaction tube. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.](image)
D. Attempt to amplify PUUV-specific RNA from patient plasma

Initial attempts were made to amplify PUUV-specific RNA from plasma samples drawn from a small group (n=9) of Finnish patients with acute HFRS caused by PUUV infection. Whole blood was drawn by venipuncture and PBMC were separated in Vacutainer CPT tubes (Becton Dickinson, Rutherford, NJ). Plasma was removed from the tubes and frozen at -70°C until use. Viral RNA was isolated and 1 μl of vRNA was added to each PCR tube. We wanted to first determine if any of the plasma samples contained viral RNA, so we did not include competitor RNA in the initial experiment. None of the plasma samples contained detectable levels of PUUV RNA (data not shown). It is possible that the virus had already been cleared in these patients or that the level of viral RNA was below the limit of detection of our assay.
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humoral cross-reactivity to the nucleocapsid protein of heterologous hantaviruses in

enzyme-linked immunosorbent assay based on recombinant proteins for sensitive and
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