Cellular Immune Response to Hantaan Virus Nucleocapsid Protein in the Acute Phase of Hemorrhagic Fever with Renal Syndrome: Correlation with Disease Severity

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Background. The cellular immune response to Hantaan virus (HTNV) is incompletely understood, especially in humans.

Methods. To investigate the cellular immunity during acute HTNV infection, the magnitude of the CD4+ and CD8+ T cell responses to HTNV nucleocapsid protein was quantitated by direct ex vivo interferon-γ (IFN-γ) enzyme-linked immunosorbent spot analysis, using an array of overlapping peptides.

Results. We found that the combined frequencies of HTNV-specific T cells at the earliest available time point (5–8 days after fever onset) were significantly higher in patients who had mild or moderate hemorrhagic fever with renal syndrome (HFRS) than in those who had severe or critical HFRS (P = .006). Moreover, these frequencies were higher in patients with subsequent mild renal failure (maximum serum creatinine level, <707 μmol/L) than in those with subsequent severe renal failure (maximum serum creatinine level, >707 μmol/L) (P = .006). Kinetic analysis showed that a decrease in the serum creatinine level during the acute phase of illness was often accompanied by an increase in the magnitude of IFN-γ-producing T cells.

Conclusion. Taken together with published data on the similar associations with neutralizing antibody, these data suggest that IFN-γ-producing T cells may help reduce the risk of progression to acute renal failure caused by HTNV infection.

Cellular immune response is believed to be crucial for the control of viral infection, and its magnitude, breadth, and quality seem to contribute to the outcome of viral infection. The roles of CD4+ and CD8+ T cells have been most clearly demonstrated during the acute phase of a few human viral infections, including those due to hepatitis C virus (HCV) [1–3] and hepatitis B virus (HBV) [4, 5].

Hantaan virus (HTNV), a member of the genus Hantavirus, causes a chronic, asymptomatic infection in its natural host, the striped field mouse Apodemus agrarius [6]. In contrast, HTNV infection in humans manifests as acute hemorrhagic fever with renal syndrome (HFRS). Clinically, HFRS is characterized by a sudden onset of high fever, followed by hypotension, oliguria, and polyuria with acute renal failure that occasionally necessitates hemodialysis treatment. Thrombocytopenia is an early abnormal laboratory finding for persons with HFRS [7]. Although the annual incidence of HFRS due to HTNV is >100,000 cases, the pathogenesis of HFRS caused by HTNV infection is poorly understood.

Nucleocapsid protein is relatively conserved among hantaviruses and is highly immunogenic in laboratory animals and humans; it has been shown to induce efficient protective immunity in animal models [8, 9]. In murine models, HTNV infection elicited a strong nucleocapsid protein–specific CD8+ T cell response 8 days after infection [10]. Furthermore, the clearance of HTNV infection depended on the appearance of functional virus-specific CD8+ T cells, which produced interferon-γ (IFN-γ) and tumor necrosis factor–α, suggesting that these cells are important for HTNV clear-
Antiviral T Cells in the Acute Phase of HFRS

**PATIENTS, MATERIALS, AND METHODS**

**Study subjects.** The study subjects comprised patients with acute HTNV infection who were recruited from Tangdu Hospital of the Fourth Military Medical University (Xi’an, China). The institutional review board of the university approved this study, and all individuals provided written informed consent. The clinical diagnosis of HFRS was confirmed by detection of IgM antibodies to HTNV in the patients’ serum specimens. Peripheral blood samples were collected from 18 patients with HFRS periodically during hospitalization. On the basis of clinical observations, illness was divided into 5 sequential stages: febrile, hypotensive, oliguric, diuretic, and convalescent.

HFRS disease severity was classified as (1) mild for patients with mild renal failure without an obvious oliguric stage; (2) moderate for those with obvious symptoms of uremia, effusion (bulbar conjunctiva), hemorrhage (skin and mucous membrane), and renal failure with a typical oliguric stage; (3) severe for those with severe uremia, effusion (bulbar conjunctiva and either peritoneum or pleura), hemorrhage (skin and mucous membrane), and renal failure with oliguria (urine output, 50–500 mL/day) for ≤5 days or anuria (urine output, <50 mL/day) for ≤2 days; and (4) critical for those with >1 of the following symptoms during severe disease: refractory shock, visceral hemorrhage, heart failure, pulmonary edema, brain edema, severe secondary infection, and severe renal failure with either oliguria (urine output, 50–500 mL/day) for >5 days, anuria (urine output, <50 mL/day) for >2 days, or a blood urea nitrogen level of >42.84 mmol/L.

**Synthetic peptides.** Seventy 15-mer peptides that overlapped by 9 amino acids and spanned the nucleocapsid protein sequence of HTNV strain 76-118 were purchased from CL Bioscientific.

**Direct ex vivo IFN-γ enzyme-linked immunosorbent spot (ELISPOT) analysis.** PBMCs were isolated by centrifugation of heparinized venous blood on Ficoll-Paque gradient and were cryopreserved until use. Seventy 15-mer peptides were pooled in 10 mixtures, each containing 7 synthetic peptides. HTNV-specific T cell responses were analyzed after overnight stimulation with individual peptides or peptide mixtures (2 × 10^5–6 × 10^5 PBMCs depleted of CD4 or CD8 by use of Dynal CD4 or CD8 beads (Dynal) were cultured overnight in the presence or absence of peptides. Spots were counted using an automated ELISPOT reader (Champ II ELISPOT reader system [Sage Creation]). The number of specific IFN-γ–secreting cells was calculated by subtracting the number of spots in unstimulated controls from that in stimulated samples. Wells with unstimulated samples never had >3 spots/well. Stimulation with 10 μg/mL of PHA (Sigma) induced vigorous responses in all samples and served as a positive control. Wells were considered positive if they yielded values >2 times greater than the background level.

**RESULTS**

**Direct ex vivo quantitation of IFN-γ ELISPOT response in patients with acute HFRS.** The overall hospitalization period...
for patients with HTNV infection was 7–30 days. In this study, mild HFRS was diagnosed in 4 patients, moderate HFRS in 4, severe HFRS in 3, and critical HFRS in 7.

The main laboratory findings are summarized in table 1. Serum creatinine concentrations were greatly elevated in all but 2 patients, and 6 patients underwent hemodialysis treatment. All patients had proteinuria. The leukocyte count was elevated in all but 5 patients, and 4 patients had high leukocyte counts, ranging from 30,300 to 85,100 cells/L. Moreover, 14 of 18 patients had thrombocytopenia during hospitalization.

The 70 peptides covering the entire HTNV nucleocapsid protein were grouped into 10 pools to map the breadth and magnitude of the T cell responses in patients with HFRS. T cell responses were studied in a cohort of 18 patients with acute HTNV infection who showed nucleocapsid protein–specific T cell responses. Of the 10 pools of synthetic peptides tested, all elicited T cell responses with different intensities, and pool 4 was able to induce IFN-γ production in 13 of 18 patients. The pools that stimulated an ELISPOT response were then screened with individual component peptides. When peptides of selected pools were retested individually, the sum of the individual peptide responses was comparable to the response against the peptide pool (data not shown).

Figure 1 shows 4 representative patients with different disease severities. In the patient who had mild HFRS, the magnitudes of the T cell responses were more vigorous than in the patients with severe or critical HFRS (figure 1), whereas the breadth of the T cell responses did not vary significantly across different disease severities (data not shown).

**Comparison of T cell responses in patients with severe or critical HFRS versus those with mild or moderate HFRS.** For statistical analysis, we used the combined frequency of T cells specific for HTNV peptides, as measured by ex vivo ELISPOT. First, we compared the combined frequencies observed at the earliest available time point during hospitalization in 11 patients from whom PBMCs were collected 5–8 days after fever onset (figure 2A). Similar trends, although not statistically significant, were observed for HTNV nucleocapsid protein–specific IFN-γ responses between patients with mild or moderate disease and patients with severe or critical disease. Because of the small numbers of ELISPOT responses observed across different disease severities, 2 groups were combined for comparison purposes: patients with mild or moderate disease formed one group, and those with severe or critical disease formed the other. During the early stage of illness, the former group displayed significantly higher T cell frequencies than the latter group ($P = .006$). Second, we compared the combined T cell frequencies in peripheral blood during the oliguric stage because we were able to collect 15 blood samples in this stage (figure 2B). Patients with mild or moderate HFRS had T cell frequencies that were significantly higher than those in patients with severe or critical HFRS ($P = .009$).

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<th>Maximum creatinine level, μmol/L</th>
<th>Minimum platelet level, $\times 1000$ platelets/μL</th>
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Kinetic analysis of T cell responses and clinical parameters of disease. In 4 patients with HFRS, we were able to collect PBMCs at ≥3 time points during hospitalization, enabling kinetic analysis of the T cell responses to HTNV nucleocapsid protein. In these patients, we compared the kinetics of the T cell responses to 2 clinical parameters of disease, serum creatinine level and platelet count (figure 3). The peak serum creatinine level was preceded by the nadir platelet count by 6–9 days. A similar pattern was seen in HFRS caused by Puumala virus infection [7]. No obvious relationship was observed between platelet counts and T cell responses. The decrease in serum creatinine level during the acute phase, however, was often accompanied by an increase in the combined frequencies of HTNV-specific T cells that produced IFN-γ in response to HTNV nucleocapsid protein peptides. Not surprisingly, a similar trend was observed for the magnitude of T cell responses to individual peptides (data not shown).

Comparison of T cell responses in patients with different serum creatinine levels. We next analyzed the relationship between the magnitude of T cell responses and the maximum
serum creatinine levels during acute HTNV infection. The combined frequencies of virus-specific T cells were considerably higher both at the earliest time point ($P = .006$) and during the oliguric stage ($P = .027$) in patients with subsequent mild acute renal failure (maximum serum creatinine level, $\geq 707 \mu$mol/L), compared with patients with subsequent severe acute renal failure (maximum serum creatinine level, $>707 \mu$mol/L) (figure 4).

**Contribution of CD4$^+$ and CD8$^+$ T cell subsets to the IFN-$\gamma$ ELISPOT response.** We were able to collect PBMCs from 12 of 18 donors after they recovered from HTNV infection 0.5–2 years later, which enabled further analysis of the T cell subsets that contributed to the IFN-$\gamma$ production detected by direct ex vivo ELISPOT analysis. PBMCs were depleted of CD4$^+$ or CD8$^+$ T cells by using antibody-coated magnetic beads, and each cell subpopulation was then separately tested in the presence of peptides. Although most IFN-$\gamma$ spots in the ELISPOT assay were abrogated with CD8$^+$ T cell depletion, a fraction of the total number of IFN-$\gamma$ spots persisted and could be attributed to CD4$^+$ T cells (figure 5). These data indirectly demonstrated that both CD4$^+$ and CD8$^+$ T cells were involved in the course of HFRS, although virus-specific CD4$^+$ T cell responses were weak, compared with CD8$^+$ T cell responses.

**DISCUSSION**

Comprehensive analysis of T cell responses during the acute phase of HTNV infection revealed a significant difference between patients with different disease severities. We demonstrated high frequencies of HTNV-specific IFN-$\gamma$-producing T cells during the acute phase of HTNV infection and reported
that, in addition to its negative association with disease severity, intense cellular immunity was associated with relatively mild renal failure (figures 2 and 4). In contrast, weak cellular immunity was associated with relatively severe renal failure. Moreover, the decrease in serum creatinine level was frequently accompanied by an increase in the magnitude of IFN-γ-producing T cells (figure 3). These data suggest that HTNV-specific IFN-γ-producing T cells may protect against severe acute renal failure caused by HTNV infection in patients with HFRS, especially during the early stage of illness. This is the first report that quantitated virus-specific T cells during acute HTNV infection and demonstrated an association between the magnitude of virus-specific T cell responses and the clinical manifestations of HFRS.

Because the present study used a panel of 15-mer HTNV peptides that could not distinguish the contribution of CD4+ T cells from that of CD8+ T cells to the overall antiviral T cell response, we determined the effects of CD4+ and CD8+ T cell depletion on the frequency of HTNV-specific IFN-γ-producing T cells. Overall, we found that CD4+ and CD8+ T cells were involved in the course of HFRS, although data obtained in blood samples collected 0.5–2 years after acute infection may not reflect the con-

Figure 4. Relationship of Hantaan virus (HTNV)-specific T cell responses to subsequent mild and severe acute renal failure (maximum serum creatinine levels during hospitalization, ≤707 and >707 μmol/L, respectively) in patients who had hemorrhagic fever with renal syndrome. A, Values for 11 patients from whom PBMCs were collected 5–8 days after fever onset. B, Values for 15 patients from whom PBMCs were collected during the oliguric stage of illness.

Figure 5. CD4+ and CD8+ T cells may contribute to interferon-γ (IFN-γ) production in response to overlapping Hantaan virus (HTNV) nucleocapsid protein peptides. PBMCs from 4 patients with different disease severities were sorted into CD4+ or CD8-depleted subpopulations, using antibody-coated magnetic beads, and stimulated separately with positive peptides to determine the number of peptide-specific, IFN-γ-producing cells.
dition during the acute phase. The magnitude of CD4+ or CD8+ T cells (figure 5 and data not shown) in patients who recovered did not appear to vary with the severity of HFRS. Furthermore, we found that HTNV-specific CD4+ and CD8+ T cells persisted for 2 years after acute infection. Previous studies have found that hantavirus-specific CD8+ T cells persist for decades [15, 17]. Similar patterns were also seen in other virus-infected patients: HCV-specific CD4+ or CD8+ T cells that produced IFN-γ persisted for 18–20 years after recovery [27]. In some patients, 2–13 years after the clinical resolution of disease, the frequency of HBV-specific T cells was comparable to that observed during the acute stage of infection [28].

Very recently, elevated levels of virus-specific CD8+ T cells correlating with disease severity were observed during the acute phase of a few human viral infections. Sin Nombre virus, another hantavirus, causes a fulminating life-threatening illness known as hantavirus pulmonary syndrome. During Sin Nombre virus infection, HLA-B*3501 is associated with severe hantavirus manifestations and the clearance of serum creatinine (figures 2 and 4), indicating that the protective functions of IFN-γ-producing T cells were similar to those of neutralizing antibodies [34]. However, the caveat must be added that this in vitro study may not exactly mirror the pathogenesis that occurs in vivo. Furthermore, despite the protective immunity elicited by the envelope glycoproteins G1 and G2 in murine models, only the cellular immune response to nucleocapsid protein, the major target of human cytotoxic T lymphocytes [15, 17], was quantitated in the present study. Because HFRS is a disease that occurs seasonally and sporadically in remote rural areas, progresses rapidly, and has severe clinical manifestations, it was a great challenge to obtain sufficient quantities of PBMCs from patients with HFRS in the acute phase for these studies. Thus far, we have been unable to obtain unambiguous evidence pointing to the conclusion that HTNV-specific IFN-γ-producing T cells primarily contribute to the protection from HTNV infection and not to the pathology of HFRS. The fine balance between protective T cell responses, which are important for viral clearance, and the large amount of virus-specific T cells with defective ability to produce IFN-γ in vitro, which may trigger immunopathology, warrants further investigation.

Acknowledgment

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References


