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A new model of Hantaan virus persistence in mice: the balance between HTNV infection and CD8$^+$ T-cell responses

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Abstract

We established a viral persistence model that involves the adoptive transfer of spleen cells from immunocompetent mice (H-2^d) into Hantaan virus (HTNV)-infected severe combined immunodeficient (SCID, H-2^d) mice. The infection is maintained despite the presence of neutralizing antibodies, without apparent signs of disease, and there is a correlation between HTNV persistence and the lack of HTNV-specific CD8$^+$ T cells. In addition, disseminated HTNV infection before the initiation of immune responses appears to be important for virus persistence. The suppression of HTNV-specific CD8$^+$ T cells in the present model appears to occur at the periphery. The present study also demonstrates that CD8$^+$ T cells contribute to the clearance of HTNV. Thus, it seems that HTNV-specific CD8$^+$ T cells play a key role in HTNV persistence in mice. This model of viral persistence is useful for studies of immune responses and immunocytotherapy against viral infection.

Keywords: Hantaan virus; Hantavirus; SCID mice; Persistent infection; CD8$^+$ T cell; Adoptive transfer

Introduction

Hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) are rodent viral zoonoses that are caused by viruses of the genus *Hantavirus*, family *Bunyaviridae* (Schmaljohn and Hjelle, 1997). At least 22 virus species have been identified (Elliott et al., 2000), which are maintained by natural rodent reservoirs despite the presence of neutralizing antibodies (Meyer and Schmaljohn, 2000). In addition, persistent hantavirus infection in natural rodent reservoirs is established during adulthood without any signs of disease (Meyer and Schmaljohn, 2000). In addition, distinct hantaviruses are associated with a single rodent species (Meyer and Schmaljohn, 2000).

To date, hantaviruses have not been found in the natural host, which is *Mus musculus* (mouse). Although Hantaan virus (HTNV), which is the prototype of the genus *Hantavirus*, is maintained in the rodent species *Apodemus agrarius* in nature (Meyer and Schmaljohn, 2000), it produces a transient infection in adult mice and lethal infection in newborn mice, following experimental infection. Therefore, studies on HTNV using experimental mice have focused on vaccine development (Choi et al., 2003) and the mechanism of viral pathogenicity (Ebihara et al., 2000; Kikuchi et al., 1998; Kim and McKee, 1985; McKee et al., 1985; Yoo et al., 1993). Previously, we established persistent HTNV infection in newborn mice by inoculation of a sublethal dose of HTNV within 24 h of birth (Araki et al., 2003). These findings suggest that the persistently HTNV-infected mouse has potential as a model of persistent viral infections, such as those caused by the human immunodeficiency virus, Epstein–Barr virus, and hepatitis B and C viruses. Persistent HTNV infection in adult mice that were infected as newborns was maintained despite the presence of high-titer neutralizing antibodies (Araki et al.,

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2003). However, we were unable to obtain unambiguous evidence that $CD8^+$ T cells contributed to the clearance of HTNV in the previous study (Araki et al., 2003) due to difficulties in modulating the immune responses of newborn mice. Therefore, a more detailed investigation of the mechanism of HTNV persistence requires a new model, that is, one in which the immune responses can be readily controlled and evaluated. In addition, the mouse model of HTNV persistence should provide information regarding the mechanism of persistent hantavirus infection in natural rodent reservoirs.

The mechanism that has been advanced for persistent lymphocytic choriomeningitis virus (LCMV) infection provides a useful platform for the evaluation of our model of HTNV persistence because cytopathic effects are either absent or incomplete in both LCMV- (Borrow and Oldstone, 1997) and HTNV-infected cells (Pensiero et al., 1992; Yanagihara and Silverman, 1990). In LCMV-infected adult mice, high-level viral replication and disseminated infection of the organs and tissues are important for the establishment of persistent infection that is accompanied by the absence of virus-specific $CD8^+$ T cells (Borrow and Oldstone, 1997). Therefore, to establish the new model of HTNV persistence, we hypothesized that high-level HTNV replication and disseminated infection of organs and tissues in mice might facilitate persistent HTNV infection that is associated with the absence of HTNV-specific $CD8^+$ T cells.

In the present study, we investigated the above-described hypothesis. We succeeded in establishing a new model of viral persistence that involves the adoptive transfer of spleen cells into severe combined immunodeficient (SCID) mice 14 days after HTNV infection. The mechanism of HTNV persistence in these mice was examined and is the basis of this report.

Results

Adoptive transfer of spleen cells to HTNV-infected SCID mice

Infection of adult SCID mice with HTNV leads to disseminated infection. In efforts to investigate whether disseminated HTNV infection before the induction of immune responses affected persistent infection, we performed the adoptive transfer of syngeneic adult immunocompetent spleen cells to HTNV-infected SCID mice, as illustrated in Fig. 1. First, we examined the spread of HTNV infection in SCID mice before the adoptive transfer of spleen cells by measuring the levels of N protein in each organ (Fig. 2). On day 7 after virus infection, N protein was detected only in the spleens. Disseminated infection was observed in various organs (except for the brain) on day 14 postinfection. On day 21 postinfection, the N protein was detected in all of the tissues examined, including the brain. The largest accumulation of N protein was found in the lungs. We then carried out the adoptive transfer of spleen cells from uninfected immunocompetent BALB/c mice into HTNV-infected, MHC-identical SCID mice (Table 1). These SCID mice, which were the recipients of immunocompetent syngeneic spleen cells on days 0, 7, 14, and 21 after virus infection, all survived (Table 1). Although the SCID mice that received syngeneic cells

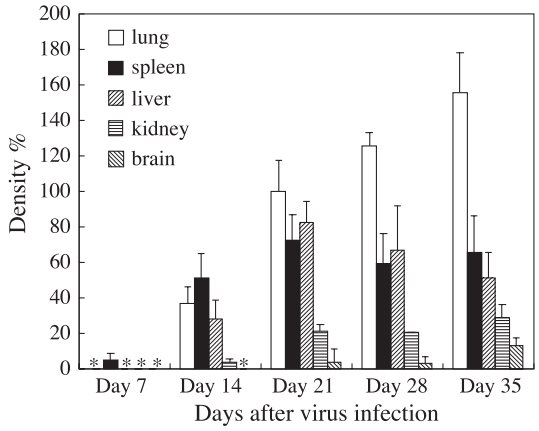

Fig. 2. Measurement of N protein levels in the organs of the HTNV-infected SCID mice. Four SCID mice per group were inoculated intraperitoneally with 2400 FFU HTNV. On days 7, 14, 21, 28, and 35 after virus infection, the lungs, spleens, livers, kidneys, and brains were obtained from the HTNV-infected SCID mice. The amount of N protein in each organ was measured by Western blotting. Band density was determined by the NIH Image 1.63 analysis software. To calculate the density of each point, the average density of the lungs of the SCID mice at day 21 after virus infection was set at 100%. The asterisks indicate that no band was detected. These results were obtained in two independent experiments. The bars represent the SD of the mean for each organ.

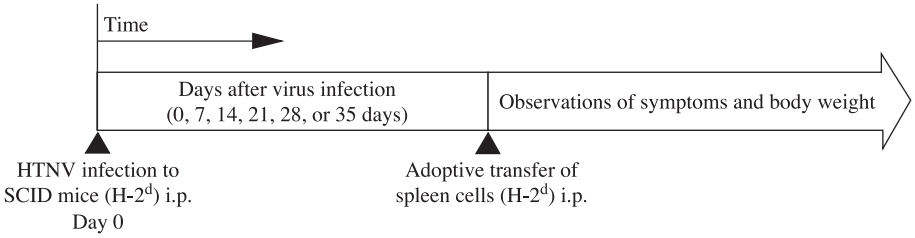

Fig. 1. Experimental design for the adoptive transfer of spleen cells. SCID mice (H-2^d) that were inoculated intraperitoneally with 2400 FFU of HTNV received 2×10^7 spleen cells from immunocompetent BALB/c mice (H-2^d) at the indicated times after virus infection. The condition of the SCID mice was observed.

Table 1
Adoptive transfer of spleen cells into SCID mice

HTNV infection before spleen cell transfer[a]	Spleen cell transfer[b] (day postinfection of transfer)	No. of mice Survivors/ tested	Time to death (days)	Outward symptoms[c]
+	+ (day 0)	4/4	–	–
+	+ (day 7)	4/4	–	–
+	+ (day 14)	4/4	–	–
+	+ (day 21)	4/4	–	+
+	+ (day 28)	2/4	44, 44	+
+	+ (day 35)	0/4	41, 44, 46, 51	+
–	+	4/4	–	–
+	–	0/4	38, 44, 46, 46	+

[a] SCID mice (H-2^d) were inoculated (+) intraperitoneally with HTNV (2400 FFU) or uninfected (–).
[b] The SCID mice either received 2×10^7 spleen cells intraperitoneally from immunocompetent (H-2^d) mice (+) or did not undergo adoptive transfer (–).
[c] Waddling gait and ruffled fur symptoms.

on days 0, 7, and 14 postinfection showed no apparent symptoms, those that received cells on day 21 demonstrated waddling gait and ruffled fur symptoms (Table 1). As shown in Table 1, 2/4 mice that received syngeneic spleen cells on day 28 and 4/4 mice that received syngeneic spleen cells on day 35 died at various time points thereafter, which suggests that the adoptive transfer of spleen cells by day 21 postinfection with HTNV is critical for survival. The body weights of the SCID mice that received spleen cells on days 0, 7, 14, and 21 postinfection were measured and compared with those of the SCID controls (no adoptive transfer after virus infection) (data not shown). The body weights of the control mice decreased from day 21 after virus infection. Mice that received splenocytes on days 0 and 7 after virus infection showed no decrease in body weight after adoptive transfer (data not shown). Although the SCID mice that received spleen cells on day 14 after virus infection showed some weight loss on day 9 after adoptive transfer (data not shown), they returned to their normal body weight shortly thereafter. On the other hand, mice that received splenocytes on day 21 after virus infection showed marked body weight loss and took a long time to return to normal body weight (data not shown). In subsequent experiments, to investigate the relationship between disseminated HTNV infection before the induction of immune responses and HTNV persistence in apparently asymptomatic SCID mice following adoptive transfer of spleen cells, we used SCID mice into which spleen cells had been transferred on days 0 and 14 after virus infection.

HTNV-specific immune responses and N protein levels in SCID mice that received spleen cells on days 0 and 14 after virus infection

Previously, we established an assay for determining the frequency of HTNV-specific CD8$^+$ T cells using intracellular gamma interferon (IFN-γ) detection (Araki et al., 2003). Using this method, HTNV-specific CD8$^+$ T-cell responses were measured on day 30 post-transfer in SCID mice that had received spleen cells by adoptive transfer on days 0 and 14 after virus infection (Fig. 3A). All of the mice that had undergone adoptive transfer on day 0 after virus infection retained HTNV-specific CD8$^+$ T cells (Fig. 3A, left panel). In contrast, HTNV-specific CD8$^+$ T cells were not detected in SCID mice that had undergone adoptive transfer on day 14 after virus infection (Fig. 3A, right panel). These results suggest that disseminated HTNV infection suppresses the induction of HTNV-specific CD8$^+$ T cells. High neutralizing antibody titers were observed in both groups (Fig. 3A). The levels of N protein were measured in the lungs. Although N protein was not detected in SCID mice that had undergone adoptive transfer on day 0 after virus infection (Fig. 3A, left panel), all of the mice that had undergone adoptive transfer on day 14 after virus infection carried N protein in their lungs (Fig. 3A, right panel). Thus, SCID mice that had received spleen cells on day 14 after virus infection showed persistent infection despite the presence of neutralizing antibodies. This SCID mouse model of persistent HTNV infection suggests that the lack of HTNV-specific CD8$^+$ T cells is important for viral persistence. These data indicate that persistent infection is possible in mice that display disseminated HTNV infection before the induction of immune responses.

Kinetics of HTNV-specific immune responses and the levels of N protein in SCID mice that had received spleen cells by adoptive transfer on day 14 after virus infection

We examined the kinetics of the HTNV-specific immune responses and the levels of N protein in SCID mice that had undergone adoptive transfer on day 14 after virus infection (Fig. 3B). On day 60 after adoptive transfer, all of the mice had N protein in their lungs and lacked HTNV-specific CD8$^+$ T cells (Fig. 3B, left panel). On day 90 after adoptive transfer, HTNV-specific CD8$^+$ T cells had appeared in 1/4 of the mice, and this was accompanied by low levels of N protein (Fig. 3B, center panel). On day 120 after adoptive transfer, the number of mice that had HTNV-specific CD8$^+$ T cells had increased, and these mice had undetectable levels of N protein in their lungs (Fig. 3B, right panel). However, even at day 120 after adoptive transfer, 1/4 of the mice continued to have N protein in the lungs and lacked HTNV-specific CD8$^+$ T cells (Fig. 3B, right panel). On the other hand, high neutralizing antibody titers were observed in all of these mice (Fig. 3B). In addition, we measured the tumor necrosis factor-alpha (TNF-α) production of the IFN-γ-producing cells upon co-culture with HTNV-infected P388D1 cells. The HTNV-specific CD8$^+$ T-cell populations in the SCID mice that had received spleen cells on days 0 and 14 after virus infection contained a significant percentage (approximately

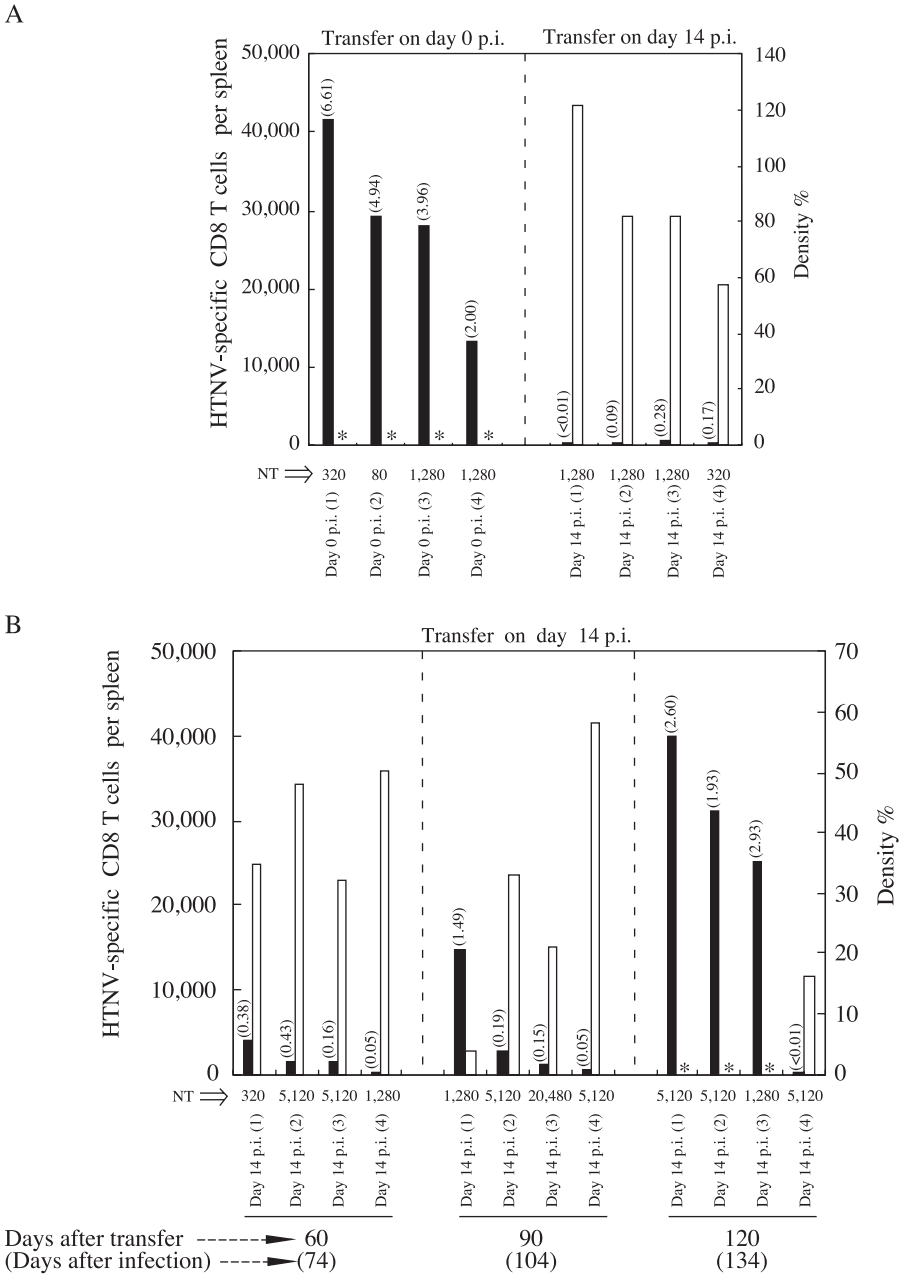

Fig. 3. Measurements of N protein levels in the lungs, neutralizing antibody titers (NT) in the sera, and IFN-γ-producing, HTNV-specific $CD8^+$ T-cell responses in SCID mice, following adoptive transfer of spleen cells on days 0 or 14 after virus infection. SCID mice were inoculated intraperitoneally with 2400 FFU of HTNV. On days 0 or 14 postinfection (p.i.), 2×10^7 spleen cells from immunocompetent mice were adoptively transferred into the HTNV-infected SCID mice. (A) On day 30 after the adoptive transfer, the lungs, sera, and spleens were removed from the SCID mice that received spleen cells on days 0 (A left) or 14 (A right) after virus infection. (B) On days 60 (B left), 90 (B center), and 120 (B right) after adoptive transfer, the lungs, sera, and spleens were removed from the SCID mice that received spleen cells on day 14 after virus infection. The black bars represent the numbers of HTNV-specific $CD8^+$ T cells that produced IFN-γ, and the white bars represent the levels of N protein. To detect HTNV-specific $CD8^+$ T cells, spleen cells and HTNV-infected P388D1 cells were co-cultured at a ratio of 1:0.5 in the presence of brefeldin A and IL-2. After a 6-h incubation, IFN-γ-producing $CD8^+$ T cells were detected by flow cytometry. The data are shown as the number of IFN-γ-producing HTNV-specific $CD8^+$ T cells per spleen. No $CD8^+$ IFN-γ^+ cells were detected from the combination of spleen cells and uninfected P388D1 cells (data not shown). The values above the black bars are the percentages of IFN-γ^+ cells among $CD8^+$ T cells. The levels of N protein in the lungs were measured by Western blotting. The band densities were determined using the NIH Image 1.63 analysis software. To calculate the density of each mouse lung, the average density of the HTNV-infected SCID mouse lung on day 21 after virus infection was set at 100%, as described in the legend to Fig. 2. The asterisks indicate where no band was detected. FRNTs were carried out using sera. The neutralizing antibody titer is expressed as the reciprocal of the highest serum dilution that resulted in a reduction of >80% in the number of infected cell foci. Uninfected SCID mice that received spleen cells lacked HTNV-specific $CD8^+$ T cells, N protein in the lungs, and neutralizing antibodies (data not shown). These results were obtained in two independent experiments.

4%) of IFN-γ-producing cells, approximately 50% of which also produced TNF-α (Fig. 4). Thus, the HTNV-specific $CD8^+$ T cells of SCID mice that had undergone adoptive transfer on day 14 after virus infection were similar, in terms of cytokine production, to those of SCID mice that had undergone adoptive transfer on day 0 after virus infection.

Detection of naïve $CD8^+$ T cells with specificity for HTNV

HTNV-specific $CD8^+$ T cells were induced in SCID mice within 30 days of the adoptive transfer of spleen cells on day 0 postinfection (Fig. 3A, left panel). Knowledge regarding the origin of these HTNV-specific $CD8^+$ T cells is important for a complete understanding of the mechanism underlying the HTNV-specific $CD8^+$ T-cell suppression that had been induced in SCID mice following adoptive transfer on day 14 after virus infection. To examine whether naïve $CD8^+$ T cells with specificity for HTNV existed in the spleen cells used for adoptive transfer, the spleen cells were transferred to HTNV-infected nude mice that lacked the thymus (Fig. 5). HTNV-specific $CD8^+$ T cells were detected in these nude mice on day 30 after adoptive transfer of the spleen cells. This result shows that naïve $CD8^+$ T cells that are specific for HTNV exist in the splenocytes that are used for adoptive transfer, and that the origin of the HTNV-specific $CD8^+$ T cells that are induced in SCID mice following adoptive transfer at day 0 postinfection is constituted by naïve $CD8^+$ T cells with specificity for HTNV. Furthermore, this result suggests that the suppression of HTNV-specific $CD8^+$ T cells seen in SCID mice that had undergone adoptive transfer on day 14 postinfection was caused at the periphery rather than in the thymus because the thymus induces tolerance during the maturation of pre-T cells, but not mature naïve T cells.

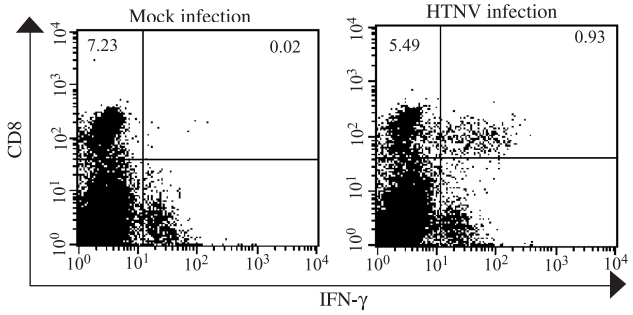

Fig. 5. Detection of naïve $CD8^+$ T cells with specificity for HTNV. Nude mice with either HTNV infection (2400 FFU) or mock infection received 2×10^7 spleen cells from immunocompetent mice on day 0 after virus infection. On day 30 after adoptive transfer, spleen cells were removed from the nude mice. The spleen cells and HTNV-infected P388D1 cells were co-cultured at a ratio of 1:0.5 for 6 h in the presence of brefeldin A and IL-2. IFN-γ-producing, HTNV-specific $CD8^+$ T cells were detected using flow cytometry. The gates were set for spleen cells, and the values shown are the percentages of IFN-γ^- $CD8^+$ and IFN-γ^+ $CD8^+$ T cells. Data from a representative experiment are shown.

$CD8^+$ T cells contribute to HTNV clearance

The results obtained, to date, have shown a correlation between HTNV persistence and the lack of HTNV-specific $CD8^+$ T cells (Fig. 3). To investigate whether $CD8^+$ T cells are required for HTNV clearance, spleen cells from HTNV-immunized immunocompetent BALB/c mice were used for the adoptive transfer (Fig. 6). These spleen cells included many HTNV-specific $CD8^+$ T cells (Fig. 6A). Whole spleen cells or the $CD8^+$ T-cell-depleted spleen cells were adoptively transferred to HTNV-infected SCID mice on day 14 after virus infection. N protein was absent from the lungs of SCID mice that had been the recipients of whole spleen cells, and numerous HTNV-specific $CD8^+$ T cells were detected (Figs. 6B, left panel, and C). In contrast, HTNV persistence was observed in SCID mice that had been

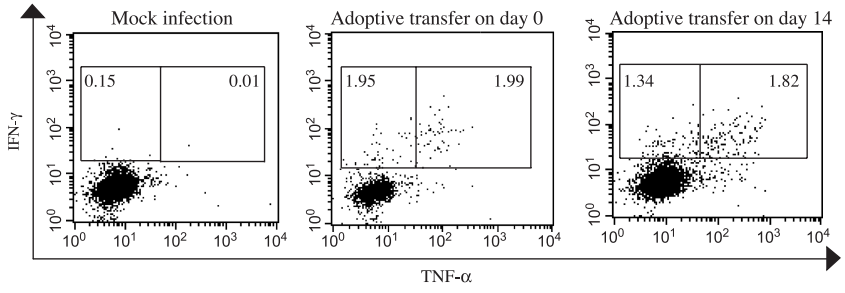

Fig. 4. TNF-α production by IFN-γ-producing $CD8^+$ T cells in splenocytes obtained from the SCID mice that received spleen cells on days 0 or 14 after virus infection. The SCID mice were infected with HTNV (2400 FFU) and received 2×10^7 spleen cells on days 0 or 14 postinfection. On day 30 after the adoptive transfer, spleen cells were obtained from the SCID mice that were adoptively transferred on day 0 postinfection. On day 120 after the adoptive transfer, spleen cells were obtained from the SCID mice that had undergone adoptive transfer on day 14 postinfection. As the control, 2×10^7 spleen cells were transferred into mock-infected SCID mice, and the spleen cells were removed from these mice on day 30 after the adoptive transfer. All of the spleen cells were tested for TNF-α production. To detect TNF-α^+ $CD8^+$ T cells, the spleen cells and HTNV-infected P388D1 cells were co-cultured at a ratio of 1:0.5 for 6 h in the presence of brefeldin A and IL-2. TNF-α production by $CD8^+$ IFN-γ^+ cells was detected using flow cytometry. The gates were set for $CD8^+$ T cells, and the values shown are the percentages of TNF-α^- IFN-γ^+ cells and TNF-α^+ IFN-γ^+ cells among $CD8^+$ T cells. Data from a representative experiment are shown.

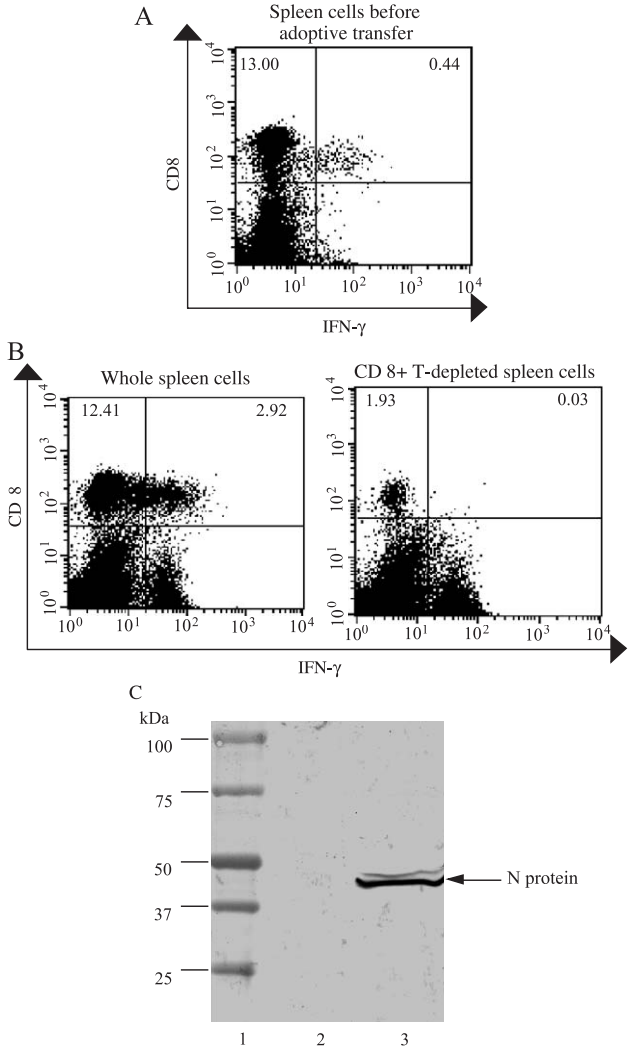

Fig. 6. The contribution of $CD8^+$ T cells to HTNV clearance. Immunocompetent BALB/c mice were inoculated intraperitoneally with 2400 FFU of HTNV. (A) On day 21 after virus infection, spleen cells were removed from the immunocompetent mice. The spleen cells and HTNV-infected P388D1 cells were co-cultured at a ratio of 1:0.5 for 6 h in the presence of brefeldin A and IL-2. IFN-γ-producing, HTNV-specific $CD8^+$ T cells were detected using flow cytometry. The gates were set for spleen cells, and the values shown are the percentages of IFN-γ^- $CD8^+$ and IFN-γ^+ $CD8^+$ T cells. (B and C) Whole spleen cells or $CD8^+$ T-cell-depleted spleen cells (>98% deletion) from the mice in (A) were transferred into SCID mice on day 14 after virus infection. On day 30 after the adoptive transfer, spleen cells and lungs were removed from the SCID mice. The methods described above for (A) were also performed to detect HTNV-specific $CD8^+$ T cells (B). The levels of N protein in the lungs were measured by Western blotting (C). Lane 1, molecular weight marker; lane 2, following the transfer of whole spleen cells; lane 3, following the transfer of $CD8^+$ T-cell-depleted spleen cells. Data from a representative experiment are shown.

recipients of $CD8^+$ T-cell-depleted spleen cells (Fig. 6C). Although the recovery of just a few $CD8^+$ T cells was observed in the SCID mice that had received the $CD8^+$ T-cell-depleted spleen cells, there was a complete absence of HTNV-specific $CD8^+$ T cells in these mice (Fig. 6B, right panel). These data show that $CD8^+$ T cells contribute to the clearance of HTNV.

Discussion

To establish a model of viral persistence in which immune responses could be readily manipulated, we carried out the adoptive transfer of spleen cells into HTNV-infected SCID mice. Persistent HTNV infection was established in SCID mice that had undergone adoptive transfer after disseminated HTNV infection of various organs. This model of viral persistence is characterized by high titers of neutralizing antibodies, no detectable levels of HTNV-specific $CD8^+$ T cells, and no apparent signs of disease. These results suggest that disseminated HTNV infection before the induction of immune responses is important for the establishment of persistent infection and for the suppression of HTNV-specific $CD8^+$ T cells in mice.

We further investigated whether the suppression of HTNV-specific $CD8^+$ T cells occurred in the periphery. Generally, clonal T-cell deletion occurs in the thymus, and there is also evidence for deletion or suppression in the periphery. In the thymus, T cells with specificities for ubiquitous self-antigens are deleted by negative selection during the process of maturation of pre-T cells, which are derived from hematopoietic stem cells (Anderson et al., 1996; Zuniga-Pflucker and Lenardo, 1996). In contrast, anergy or clonal deletion of naïve T cells with specificities for self-antigens that do not exist in the thymus take place in the periphery. Nude mice, which lack the thymus, were used to detect naïve $CD8^+$ T cells with specificity for HTNV in the spleen cells that were used for adoptive transfer. Because the thymus is required for the maturation of pre-T cells that are derived from hematopoietic stem cells, nude mice are unable to make T cells from hematopoietic stem cells or pre-T cells. The nude mice that received spleen cells on day 0 after HTNV infection had significant numbers of HTNV-specific $CD8^+$ T cells (Fig. 5). Consequently, the origin of the HTNV-specific $CD8^+$ T cells that were induced in the nude mice was naïve $CD8^+$ T cells, and not hematopoietic stem cells. These findings suggest that naïve HTNV-specific $CD8^+$ T cells existed in the spleen cells used for adoptive transfer, and that these T cells experienced anergy or clonal deletion in the periphery of SCID mice that had undergone adoptive transfer on day 14 after virus infection. These results also suggest that it is possible to establish persistent HTNV infection, not only in newborn mice that have immature immune systems, as shown previously (Araki et al., 2003), but also in adult mice that possess mature immune systems.

In a previous study of HTNV-infected newborn mice (Araki et al., 2003), we were unable to demonstrate conclusively that $CD8^+$ T cells contributed to HTNV clearance due to difficulties in modulating the immune responses of

the newborn mice. In our present study, HTNV persistence was observed in SCID mice that were recipients of $CD8^+$ T-cell-depleted syngeneic spleen cells, obtained from HTNV-immunized immunocompetent mice. In contrast, SCID mice that were recipients of unfractionated spleen cells from HTNV-immunized mice showed an absence of detectable N protein (Fig. 6). This demonstrates that $CD8^+$ T cells contribute to HTNV clearance. Additionally, it is known that HTNV-specific $CD8^+$ T cells are induced in both HTNV-infected humans and mice (Araki et al., 2003; Park et al., 2000; Van Epps et al., 1999), and that these infections are transient. In addition, hantavirus-specific $CD8^+$ T cells have been observed in other transient hantavirus (Puumala virus and Sin Nombre virus) infections of humans (Ennis et al., 1997; Van Epps et al., 2002). Taken together, it seems that $CD8^+$ T cells are required for the clearance of hantaviruses. These results also suggest that persistent hantavirus infections in natural rodent reservoirs are due to escape from $CD8^+$ T cell immune surveillance, although it is unclear how hantaviruses escape the hantavirus-specific $CD8^+$ T cells in natural rodent reservoirs.

In addition to the mechanism of HTNV clearance, the mechanism of HTNV persistence is a key issue in this mouse model. HTNV-infected SCID mice that did not receive syngeneic spleen cells died from the infection, and HTNV persistence was established in HTNV-infected SCID mice that received $CD8^+$ T-cell-depleted syngeneic spleen cells from HTNV-immunized immunocompetent BALB/c mice. These results indicate that $CD4^+$ T cells and B cells contribute to viral persistence in the absence of any signs of disease.

However, B cells seem to be more important than $CD4^+$ T cells for HTNV persistence in mice. Nude mice showed asymptomatic viral persistence when infected intraperitoneally with HTNV (K. Yoshimatsu, and J. Arikawa, unpublished data). The persistently HTNV-infected nude mice had low titers of HTNV-specific antibodies. These results suggest that the B cells and low-titer antibodies that are induced without $CD4^+$ T-cell help are important for HTNV persistence in mice. $CD4^+$ T cells may simply play a supporting role in the production of high-titer antibodies that are specific for HTNV.

Moreover, it seems that the role of HTNV-specific antibodies that are produced by the B cells in persistently HTNV-infected mice is to evade fatal disease. Generally, the high-level growth of HTNV in the brain is important in producing the fatal outcome in mice (Ebihara et al., 2000; Kurata et al., 1983; McKee et al., 1985). The passive transfer of HTNV-specific antibodies into infected mice confers protection against lethal infection (Arikawa et al., 1992; Yoshimatsu et al., 1993). Therefore, antibodies are important for the suppression of HTNV growth in the brain and in the evasion of fatal illness. These findings suggest that HTNV-specific antibodies are necessary for HTNV persistence in the absence of morbidity. Thus, the existence of B cells is essential for the establishment of persistent HTNV infection without any signs of disease in mice.

Additionally, NK cells are known to contribute to innate defense against viral and bacterial infections. SCID mice have mature NK cells that are phenotypically and functionally indistinguishable from NK cells found in immunocompetent mice (Kumar et al., 1989). However, because the HTNV-infected SCID mice died from the infection, it is clear that NK cells alone are not sufficient to confer protective immunity against HTNV infection. However, it remains unclear whether NK cells act cooperatively with other components of the immune system to generate protective immunity.

The present model of viral persistence is similar, in terms of the presence of neutralizing antibodies and the lack of apparent signs of disease, to natural rodent reservoirs that are persistently infected with hantaviruses (Meyer and Schmaljohn, 2000). Although it is believed that persistent hantavirus infection in natural rodent reservoirs is life-long (Meyer and Schmaljohn, 2000), HTNV was eliminated by day 120 post-transfer from most of the SCID mice that had undergone adoptive transfer on day 14 after virus infection. In this respect, it appears that the present model does not reflect, completely, the natural rodent reservoir. Therefore, comparisons of the HTNV-specific T-cell responses between the present model and the natural rodent reservoir need to be made within the context of these findings, and the results should be important with regard to understanding persistent hantavirus infection in nature. However, this type of comparison is difficult because, to the best of our knowledge, there have been no reports on the T-cell responses in hantavirus-infected natural rodent reservoirs. To further investigate the mechanism of persistent hantavirus infection in natural rodent reservoirs, the establishment of a method for measuring hantavirus-specific $CD8^+$ T cells in natural rodent reservoirs is required.

When the spleen cells were adoptively transferred into HTNV-infected SCID mice on day 21 after virus infection, an exacerbation of disease symptoms was observed, as compared to HTNV-infected SCID mice that did not undergo adoptive transfer. Previously, we showed an increase in the serum BUN levels of HTNV-infected SCID mice following adoptive transfer (Yoshimatsu et al., 1997). These results suggest that immune system-mediated pathogenicity occurred in these mice. In addition, both HFRS and HPS patients demonstrate varying degrees of pathology that are thought to be immune-related because hantaviruses infect endothelial cells without direct cytopathic effects (Pensiero et al., 1992; Sundstrom et al., 2001; Zaki et al., 1995), and this is accompanied by marked increases in both $CD8^+$ T-cell numbers and in the levels of inflammatory cytokines, such as IFN-γ, TNF-α, and IL-6 (Chen and Yang, 1990; Huang et al., 1994; Krakauer et al., 1994; Linderholm et al., 1996; Markotic et al., 1999; Temonen et al., 1996). Therefore, the adoptive transfer on day 21 after virus infection of

SCID mice may have potential as a model of HFRS and HPS.

In summary, we have established a model of viral persistence using SCID mice and HTNV. It seems that disseminated HTNV infection before the induction of immune responses plays a key role in the persistent infection and suppression of the generation of HTNV-specific $CD8^+$ T cells from the naïve $CD8^+$ T cells of mice. Furthermore, we have demonstrated that $CD8^+$ T cells contribute to HTNV clearance. The present model of viral persistence is useful for studies of immune responses and of immunocytotherapy against viral infection because the induction of immune responses, and the types of immunocytes used for adoptive transfer, can be readily manipulated.

Materials and methods

Mice

Six-week-old BALB/c mice (H-2^d) and six-week-old BALB/c-nu/nu mice (nude mice, H-2^d) were obtained from SLC (Hamamatsu, Japan). Six-week-old CB-17/Lcr-scid Jcl mice (SCID mice, H-2^d) were obtained from CLEA Japan (Tokyo, Japan). All of the mice were treated according to the laboratory animal control guidelines of our institute, which conform to those of the U.S. National Institute of Health. All of the experiments were carried out in a class P3 facility.

Viral infection of mice

HTNV cl-1 (Ebihara et al., 2000) was obtained by plaque cloning of HTNV strain 76-118. The virus was propagated in the E6 Vero cell clone (Vero E6), which was grown in Eagle's minimal essential medium (EMEM; Invitrogen) that was supplemented with 5% fetal bovine serum (FBS). Six-week-old adult BALB/c, nude, and SCID mice were inoculated intraperitoneally with 2400 focus-forming units (FFU) of HTNV.

Cell preparations

Single-cell suspensions of spleen cells were obtained by homogenizing spleens through a mesh. The erythrocytes were lysed with 0.83% NH_4Cl. For the adoptive transfer experiments, the spleen cells were suspended in RPMI 1640 medium (Invitrogen) that was supplemented with 10% FBS and 50 μM 2-mercaptoethanol (ME) and incubated in a 150-cm^2 cell culture flask for 2 h at 37 °C in 5% CO_2 to remove adherent cells. To deplete the $CD8^+$ T cells, the spleen cells (1×10^7 cells/ml) were treated with the rat anti-mouse CD8 monoclonal antibody (mAb, 1 μg/ml; Serotec) or rat IgG (isotype control, 1 μg/ml; Serotec) for 1 h on ice, and then incubated in a 1:10 dilution of rabbit complement (Low-Tox-M; Cederlane Laboratories, Hornby, ON, Canada) for 1 h at 37 °C. Flow cytometric analysis was performed to determine the efficacy of the procedure; >98% of the $CD8^+$ T-cell subpopulation was depleted.

Adoptive transfer of spleen cells into SCID or nude mice

Spleen cells were obtained from naïve BALB/c mice or HTNV-infected BALB/c mice, 21 days after infection. The SCID and nude mice received either 2×10^7 spleen cells or 1.7×10^7 $CD8^+$ T-cell-depleted spleen cells by intraperitoneal injection.

Clinical features of the mice

Waddling gait, ruffled fur, weight loss, and mortality were used to evaluate the clinical severity of HTNV infection.

Fluorescence staining and flow cytometry

To detect HTNV-specific $CD8^+$ T cells, we used flow cytometry to assay the intracellular cytokines of $CD8^+$ T cells that were stimulated by HTNV-infected antigen-presenting cells, as described previously (Araki et al., 2003). Briefly, spleen cells were added to 96-well round-bottomed plates at a concentration of 5×10^5 cells/well in RPMI 1640 medium that was supplemented with 10% FBS, 50 μM 2-ME, 20 U/ml recombinant interleukin (IL)-2 (Sigma Co., St. Louis, MO), and 10 μg/ml brefeldin A (Sigma), along with the HTNV-infected or noninfected P388D1 cells at a concentration of 2.5×10^5 cells/well. After a 6-h incubation, the cells were stained with Tri-color (TC)-conjugated rat anti-mouse CD8a (Ly-2) mAb (Caltag Laboratories, San Francisco, CA) for 30 min on ice, then fixed and permeabilized with saponin (Sigma), before the addition of the fluorescein isothiocyanate-conjugated rat anti-mouse IFN-γ mAb (Caltag Laboratories) and the *R*-phycoerythrin-conjugated rat anti-mouse TNF-α mAb (Caltag Laboratories). The cell samples were analyzed using the FACSCalibur system (Becton Dickinson), and the data analyses were conducted with CellQuest (Becton Dickinson).

Western blotting for the detection of N protein in lung, liver, spleen, kidney, and brain tissues of HTNV-infected mice

Western blotting was performed using previously published methods (Yoshimatsu et al., 1995). Briefly, 20 μl of 10% tissue homogenate was used as the antigen. Polyclonal rabbit anti-N protein antibody (diluted 1:800 with PBS) (Araki et al., 2001), which was prepared by immunizing a rabbit with the truncated N protein expressed in *E. coli*, was used to detect the N protein on the membrane. Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:500, Jackson ImmunoResearch, West Grove, PA) was used as the sec-

ondary antibody. Western blotting was quantified by densitometry with the NIH Image v. 1.63 analysis software. NIH Image was developed at the National Institute of Health and is available on the Internet (http://rsb.info.nih.gov/nih-image/).

Focus reduction neutralization test

Focus reduction neutralization tests (FRNTs) were performed using the previously published method (Araki et al., 2001). Briefly, 100 μl of serial 4-fold dilutions of serum that had been heat-inactivated for 30 min at 56 °C were mixed with equal volumes of virus suspensions that contained 400 FFU of HTNV and incubated at 37 °C for 1 h. Then, 50-μl aliquots of the mixture were inoculated onto Vero E6 cell monolayers in 96-well plates and incubated at 37 °C for 1 h in a 5% CO_2 incubator. After adsorption for 1 h, the wells were overlaid with EMEM that contained 1.5% carboxymethyl cellulose. The plates were incubated for 7 days, and the monolayers were fixed with acetone/methanol (1:1) and dried. For the detection of virus foci, the polyclonal rabbit anti-N protein antibody was added to the 96-well plate. The plate was incubated for 1 h at 37 °C, washed with PBS, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:500; Jackson ImmunoResearch) for 1 h at 37 °C. The plate was washed with PBS, and the viral foci were stained with the 3-amino-9-ethylcarbazole substrate (Sigma), according to the manufacturer's instructions.

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