Hantavirus Infection Induces a Typical Myocarditis That May Be Responsible for Myocardial Depression and Shock in Hantavirus Pulmonary Syndrome

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Despite clinical evidence of myocardial dysfunction, there is no pathological evidence of myocardial injury in hantavirus pulmonary syndrome (HPS). The dominant opinion is that the primary cardiac lesion is functional rather than structural. The present study describes hantaviral antigen and particles in the cardiac endothelium and interstitial macrophages in association with a typical myocarditis in HPS. Human hearts from 14 individuals who died of HPS were compared with hearts from 14 individuals who died of acute necrotizing pancreatitis associated with acute lung injury and 4 individuals who died accidental deaths without thoracic injury (as controls); all cases were selected from autopsies. Transmural blocks of myocardial tissue were excised from the middle portion of the left-ventricular free wall and fixed in formalin. Small samples of myocardial tissue from 4 HPS cases and 4 non-HPS controls were fixed in glutaraldehyde for electron microscopic study. Histomorphometric, immunohistochemical, and ultrastructural methods were employed to detect the presence of hantavirus in the myocardium and to evaluate interstitial edema and the minor diameter of myocytes, to characterize the immunophenotype, and to estimate the number of inflammatory cells in and situ cytokine-producing cells and the T helper cell subset 1 and 2 immune responses (tumor necrosis factor [TNF]–α, interferon-γ, interleukin [IL]–10, and IL-4). Cardiac remodeling; hantaviral antigen and particles in the endothelium and macrophages; scattered foci of myofiber necrosis; greater interstitial cellular infiltration, mainly composed of macrophages and memory T lymphocytes and a significant number of T helper and B lymphocytes; and TNF-α protein expression in macrophage-type cells and cardiomyocytes were observed to a greater extent in HPS myocardium than in normal and acute pancreatitis control myocardium. These findings give support to the opinion that structural changes could be responsible for myocardial depression and shock in HPS, and it should be properly named as “hantavirus cardiopulmonary syndrome” (HCPS).

Hantaviruses are enveloped viruses with a negative-sense, single-stranded RNA genome that belong to the family Bunyaviridae [1, 2]. Distinct hantavirus types are distributed throughout the world and are associated with different primary rodent reservoirs [1, 3–5]. The spectrum of clinical symptoms caused by hantavirus infections in humans varies from subclinical presentation to pulmonary involvement progressing with shock (hantavirus pulmonary syndrome [HPS]) or severe hemorrhagic fever with renal involvement (hemorrhagic fever with renal syndrome) [6–9].

HPS is an acute respiratory illness, first identified in Four Corners, southwestern United States, in 1993.
characterized by a capillary-leak syndrome in the lungs and clinically presenting as an adult respiratory distress syndrome [9–11]. It was first described as being caused by Sin Nombre virus (SNV) infection, with prodromes of fever, myalgia, and headaches, followed by rapidly progressive pulmonary edema and hemorrhage, circulatory shock, and death in most of the cases [10, 11]. At present, it is well known that several species of the hantavirus genus have wide distribution in the Americas, causing HPS with similar clinical manifestations [12, 13]. Inflammatory cytokines produced locally after activation of lymphomononuclear cells in the lungs and systemic production of tumor necrosis factor (TNF–α), interleukin (IL)–10, IL-4, and interferon (IFN)–γ by activated mononuclear cells have been suggested to play a role in the pathogenesis of HPS [14, 15]. SNV virions and antigen were identified in endothelial cells in the lungs and other organs in fatal cases of HPS [10, 11].

Despite clinical evidence of myocardial dysfunction [7, 16, 17], there is no pathological evidence of myocardial injury [10, 11, 18]. The dominant opinion is that the primary cardiac lesion in HPS is functional rather than structural. The present study describes hantavirus antigen and particles in the cardiac endothelium and interstitial macrophage-type cells in association with a typical myocarditis in 14 fatal cases of HPS.

MATERIALS AND METHODS

Specimens. Human hearts from 14 individuals who died of HPS in the acute phase (8 men and 6 women; 16–50 years of age) obtained from the Death Verification Service (DVS), University of São Paulo, Ribeirão Preto, from 1999 to 2002 were studied. The diagnosis of hantavirus infection was confirmed by detection of the antibody (IgM) to SNV antigen by ELISA in postmortem blood samples and by immunohistochemical staining of hantavirus antigen in body tissues [11, 19]. Hantavirus gene sequences were studied by nested reverse-transcription (RT)–polymerase chain reaction (PCR) in postmortem blood samples from only 7 cases [13], because blood was not available from all HPS autopsy cases at that time. Eighteen human hearts from non-HPS cases were used as controls: 14 individuals with acute pancreatitis-associated lung injury (APALI) (12 men and 2 women; 24–54 years of age) and 4 individuals who died accidental deaths without thoracic injury (non-APALI) (4 men; 16–40 years of age) obtained from the DVS and the Medico-Legal Institute (Ribeirão Preto, São Paulo), respectively. All hearts were obtained 1.5–28 h after death. The hearts were removed, washed, blotted, and weighed. Because body weight is an important source of variability in organ weight, the predicted heart weight was calculated by use of the results of a previous study on body weight–related changes in normal human heart weight [20]. A cross-section of the entire heart was made, perpendicular to the left-ventricular axis at the level of the papillary muscles (medioventricular). Myocardial samples were excised from the left-ventricular free wall, halfway between the base and the apex, and were prepared for light microscopy (hematoxylin-eosin [HE] and immunohistochemistry) and electron microscopy.

Immunohistochemistry. The avidin-biotin-peroxidase–complex and streptavidin-biotin enzyme complex immunostaining methods were used with antibodies against CD4 T helper lymphocytes (monoclonal antibody [MAb] mouse anti-human CD4; 1F6 clone; 1:60; Novoceastra), CD8 T lymphocytes (MAb mouse anti-human CD8; 4B11 clone; 1:200; Novoceastra), B lymphocytes (MAb mouse anti-human CD20; L26 clone; 1:1500; Dako), memory T lymphocytes (MAb mouse anti-human CD45RO; UCHL-1 clone; 1:600; Dako), macrophages/monocytes (MAb mouse anti-human CD68; KP1 clone; 1:6000; Dako), IL-4 (MAb goat anti-human IL-4; AB-204-NA; 1:30; R&D Systems), IL-10 (MAb mouse anti-human IL-10; MAB217; 1:10; R&D Systems), IFN–γ (MAb mouse anti-human IFN–γ; MAB285; 1:100; R&D Systems), and TNF–α (polyclonal rabbit anti-human TNF–α; IP300; 1:200; Genzyme). Immunohistochemical reactions were performed on 4-μm-thick sections in accordance with the manufacturers’ directions. Diaminobenzidine was used as the color substrate, and Meyer’s hematoxylin was used as counterstaining. For immunostaining of hantaviral nucleoprotein antigens, MAb mouse anti-Puumala nucleoprotein (GB04-BF07 clone; 1:200; Centers for Disease Control and Prevention) was used, followed by sequential application of rabbit anti-mouse link antibody, alkaline phosphatase anti–alkaline phosphatase (APAAP) complex, and naphthol/fast red substrate (Dako Corporation). Briefly, epitope retrieval was performed by boiling 10 mmol/L (pH 6.0) citric acid solution in a pressure cooker for 3 min. The MAb was incubated overnight at 4°C. This was followed by sequential application of rabbit anti-mouse link antibody, APAAP complex, and naphthol/fast red substrate. Sections were then quickly counterstained with Meyer’s hematoxylin. For the creation of negative controls, primary antibodies were omitted during the staining process.

Quantification of stained cells. Cell immunophenotypes, cytokine-producing cells, and hantavirus-positive host cells (endothelial cells and macrophage-type cells) were identified and quantified using a light microscope. At least 20 areas of sub-endocardial and midmyocardial zones of the left-ventricular free wall were randomly selected for analysis under ×400 magnification, and the number of positive cells per square millimeter was determined.

Morphometric quantification of myocardial edema. To estimate the volume fraction (%) of the interstitial space in HE-stained sections, quantitative examination of the left-ventricular myocardium was performed on a medium-power light-microscopic field (×400) with a 100-point Ocular Integration Eyepiece II (Carl Zeiss). For each heart, 5 fields of the midmyocardial zone were randomly selected and analyzed, and the
volume fraction of the interstitial space per square millimeter was calculated.

**Histomorphometric analysis of myofiber diameter.** The minor diameter of myocytes was measured in HE-stained sections on a medium-power light-microscopic field (×400). The diameter of subendocardial fibers was not estimated, because degenerative changes predominate in the subendocardial layers as a result of the imbalance between demand for and availability of oxygen. For each case, 20 longitudinally oriented myofibers were randomly selected, and the transversal diameter through the nucleus was estimated. Measurements were made by a skilled observer blinded to the groups. Findings were averaged for each group. Morphometric analysis was performed using videomicroscopy with Leica Qwin software (version 3.2.1; Leica Imaging Systems) in conjunction with a Leica microscope DMR (Leica Microsystems), a videocamera (Leica Microsystems), and an online computer.

**Transmission electron microscopy.** Small blocks (1 mm³) of myocardial tissue from 4 HPS cases and 4 non-HPS controls were fixed in 2% glutaraldehyde/2% paraformaldehyde in cacodylate buffer overnight, postfixed in 1% osmium tetroxide, dehydrated, and embedded in araldite. Ultrathin sections obtained from selected areas were double-stained and examined in a Zeiss EM109 electron microscope (Carl Zeiss) at 80 kV.

**Statistical analysis.** The number of cell immunophenotypes and cytokine-producing cells in tissues from patients with HPS and control subjects was compared by use of the Kruskal-Wallis test and the Dunn test for multiple comparisons. The Pearson test and nonparametric Spearman test were used for correlation of variables with normal and abnormal distributions, respectively. \( P < .05 \) was considered to be statistically significant. Unless specified otherwise, data are presented as mean ± SD.

**RESULTS**

**Clinical findings.** At arrival in the hospital, all patients with HPS presented with acute respiratory distress syndrome (ARDS), tachycardia, and low blood pressure. Five patients died within 1 h after admission. The remaining 9 patients showed bilateral diffuse interstitial and alveolar infiltrates on chest radiograph and hypoxemia with low \( O_2 \) saturation and acidosis. Clinical
parameters of heart failure, apart from diffuse leak pulmonary edema, were noted in the medical records of these patients, including tachycardia, low blood pressure, and renal dysfunction. Available laboratory data showed increased hematocrit (57.8% ± 5.2%; range, 50%–63%) and thrombocytopenia (51 × 10^3 – 36 × 10^3 platelets/mm^3; range, 16 × 10^3–117 × 10^3 platelets/mm^3) in 6 of 8 patients with HPS tested. Neutrophilic leukocytosis with a shift to the left (i.e., an increased number of immature neutrophils in the circulation) was reported in 7 of 8 patients tested. Proteinuria (mild to severe [1+ to 3+/4+]) and increased serum creatinine levels (mg/dL; range, 2.0–1.4–2.8 mg/dL) were seen in 6 of 8 patients with HPS tested. The creatinine kinase–MB fractions in the blood serum were >25 U/L in 2 patients tested (64 and 319 U/L). The APALI control lungs (n = 14) showed acute injury patterns at autopsy, consistent with clinical ARDS. The non-APALI control lungs (n = 4), also collected at autopsy, were normal.

Pathological changes. Lungs from HPS cases and APALI controls appeared firm, airless, and “beefy” on gross examination. Microscopically, marked interstitial and intra-alveolar edema associated with mononuclear cell inflammatory infiltrate, minimal epithelial injury, and a delicate meshwork of fibrin and hyaline membranes within the alveolar spaces could be observed. The non-APALI control group showed normal lungs.

All HPS hearts showed flabby walls and mild biventricular dilatation. No signs of cardiac dilatation or heart failure could be seen in APALI or non-APALI control hearts. The mean heart weight for HPS cases (334.30 ± 63.06 g) was not different from the predicted heart weight for patients of the same body weight (321.50 ± 46.95 g). A mild to moderate edematous interstitial inflammatory process with variable congestion and mononuclear cell interstitial infiltrate was observed in all HPS myocardia (figure 1A). This inflammatory infiltrate was composed of small

Figure 2. Representative features of hantavirus pulmonary syndrome (HPS) myocardium showing interstitial inflammatory infiltrate mainly composed of CD68 macrophages (A) and CD45RO T lymphocytes (B). Scale bar, 50 μm. The numbers of CD68 macrophages (C), CD45RO T lymphocytes (D), CD4 T lymphocytes (E), and CD20 B lymphocytes (F) in HPS myocardium are significantly higher than the numbers found in acute pancreatitis–associated lung injury (APALI) and non-APALI myocardium. No statistically significant differences can be observed in the number of CD8 T lymphocytes in the 3 groups (G).
and large mononuclear cells. Histomorphometric analysis to characterize the interstitial edema demonstrated that the volume fraction (%) of interstitial space in HPS myocardium was significantly higher: 45.30% ± 9.24%/mm², vs. 27.06% ± 9.49%/mm² and 26.35% ± 8.60%/mm² in APALI and non-APALI controls, respectively (figure 1B). The myocytes appeared diffusely “overstretched and wavy” in small clusters. The mean minor diameter of myocytes in HPS myocardium was significantly smaller: 13.22 ± 1.69 μm, versus 17.59 ± 3.41 and 20.60 ± 1.81 μm in APALI and non-APALI controls, respectively (figure 1C). Coagulative myocytolysis with a basophilic cytoplasm and cariopnecnosis could be seen in scattered myocytes in HPS hearts (figure 1D).

Inflammatory cell immunophenotypes. The myocardial interstitial inflammatory infiltrate in HPS hearts was mainly composed of CD68 macrophages (figure 2A and 2C) and a significant number of CD45RO T lymphocytes (figure 2B and 2D), CD4 T lymphocytes (figure 2E), and CD20 B lymphocytes (figure 2F). The interstitial macrophages in HPS hearts appeared to be larger and more elongated than those found in APALI and non-APALI control hearts and were diffusely intermingled in the perimysium and endomysium; they were particularly concentrated around intramyocardial vessels. No statistically significant differences were observed in the number of CD8 T lymphocytes in the 3 groups (figure 2G).

Cytokine expression. All HPS cases showed markedly stained large macrophages expressing TNF-α protein (figure 3A and 3B), and 6 of the 14 cases showed expression of TNF-α, with staining localized to multiple patches of cardiomyocytes (figure 3B). The mean number of cells expressing TNF-α protein per square millimeter of tissue in HPS hearts was significantly higher than that in APALI and non-APALI control hearts (figure 3C). Although cells expressing IL-10 and IL-4 were found in HPS hearts, there was no statistically significant difference in comparison with the values in APALI and non-APALI control hearts (data not shown). The numbers of macrophage-type cells expressing TNF-α per square millimeter in HPS hearts were positively correlated with the numbers of macrophage-
type cells expressing IL-10 (figure 3D). INF-γ-producing cells were not immunohistochemically detected in HPS hearts or in APALI and non-APALI control hearts.

**Immunostaining of hantavirus antigens.** Immunohistochemical evidence for the presence of hantaviral antigen was documented in 13 HPS myocardia (92.86% of HPS hearts), in the cytoplasm of intramyocardial endothelial cells and interstitial macrophage-type cells. The numbers of cells staining positively for hantavirus in HPS myocardium were positively correlated with the numbers of macrophages (figure 4A) and with the numbers of cells expressing TNF-α protein in these hearts (figure 4B). The immunohistochemically stained hantaviral antigen appeared as a dotlike and finely granular pattern in the cell cytoplasm (figure 5A).

**Transmission electron microscopy.** The electron microscopic study of HPS myocardial samples disclosed typical granulofilamentous hantavirus inclusions of N nucleoprotein within endothelial cells and macrophage-type cells. They varied in shape from small and spherical to more oblong. Also, multiple hantavirus-like particles were seen within vacuoles in the cytoplasm of endothelial cells and macrophage-type cells. Swollen endothelial cells showed hantavirus-like particles intermingled with N nucleoprotein inclusion bodies (figure 5D). The ultrastructural features of the myocardial cells in HPS, APALI, and non-APALI hearts were disregarded because the specimens were obtained after death.

**DISCUSSION**

A fundamental question is that of the potential role of cardiac changes in the pathogenesis of HPS shock. There are 3 major findings in present study. First, the myocardium in fatal HPS cases showed endothelial cells and interstitial macrophage-type cells harboring hantavirus antigen and viral particles. Second, a typical myocarditis could be seen in HPS hearts, with an inflammatory interstitial infiltrate composed of mononuclear cells; this was not seen in samples taken from normal non-APALI control hearts and from control hearts taken from patients who died of APALI. Third, hearts of patients who died of HPS shock were remodeled, and macrophages in the inflammatory infiltrate and cardiomyocytes expressed TNF-α.

**The heart as a primary target of hantavirus infection.** Infection with hantavirus was confirmed in all 14 HPS cases studied by ELISA and immunohistochemistry. The hantavirus gene sequences determined by nested RT-PCR in 7 cases showed a high degree of homology with the Araraquara virus (data not shown), a strain of higher prevalence in southeastern Brazil [12, 13]. Moreover, macrophage-type cells and endothelial cells—but not cardiomyocytes—in HPS hearts harbored immunostained hantiviral N nucleoprotein antigen. This contrasts with previous reports describing random immunostaining positivity to N nucleoprotein epitopes in intramyocardial endothelial cells [10, 11]. The nucleoprotein N of HPS-associated hantaviruses was identified by immunohistochemistry for the first time in pulmonary endothelial cells and alveolar macrophages [7, 10, 11]. Next, a few of these host cells have also been immunostained with MAb mouse anti-Puumala nucleoprotein in a wide range of human organs [3, 10, 11]. To exclude the possibility that the immunolocalization of the hantaviral antigens in both endothelial cells and macrophage-type cells could represent either phagocytosis of extracellular antigens or entrapped virions, HPS myocardium samples were studied by transmission electron microscopy, which showed hantavirus particles within endothelial cells and macrophage-type cells, including viral particles budding into endothelial vesicles or cisternae and into the blood. Furthermore, classic granulofilamentous viral inclusions inside of endothelial cells were seen. These ultrastructural findings are in accord with the hantaviral morphology reported in tissue culture and lungs from fatal cases of HPS [6, 10, 11, 21]. The sequence of events from inhalation of infectious viral particles until the onset of pulmonary capillary leakage and systemic spread of the virus is poorly known [22].

**Evidence for the existence of a typical myocarditis in HPS.** Myocarditis is an inflammatory disease of the myocardium that must be diagnosed by established histological, immunological,
and immunohistochemical criteria [23]. According to the Dallas criteria, early and definitive diagnosis of myocarditis depends on the detection of an inflammatory infiltrate in the myocardium [24, 25]. Because the Dallas criteria have been developed to be applied to small endomyocardial biopsy specimens, the routine application of immunohistochemistry and PCR analysis for characterization of the inflammatory cell infiltrate and identification of infective agents has become essential for the diagnosis of myocarditis, to overcome the limits of the histopathological Dallas criteria [23].

In the present study, an edematous interstitial myocarditis with a mononuclear inflammatory infiltrate of mononuclear cells, mainly macrophages, with larger and more elongated morphologic features suggestive of “activation” and memory T lymphocytes was observed in HPS hearts, whereas this was not seen in control hearts. Also, scattered foci of cardiomyocyte necrosis associated with the interstitial inflammatory infiltrate were noted in HPS hearts. In addition, a significant number of T helper (CD4) and B (CD20) lymphocytes could be characterized. These findings are in agreement with the conclusion of the 1997 International Society and Federation of Cardiology task force that ≥14 lymphocytes (mainly CD45RO T lympho-
cytes) or macrophages per square millimeter of myocardium is a reliable threshold for the diagnosis of myocarditis in the appropriate clinical context [26]. Furthermore, the histological diagnosis of myocarditis caused by hantavirus is corroborated by the finding of clusters of macrophage-type cells with foci of cardiomyocyte necrosis, similar to those described in an experimental model of Coxsackievirus B3–induced myocarditis in genetically immunodeficient mice [27]. It is known that macrophages are important effector cells implicated in many cardiac diseases, including atherosclerosis, sepsis/septic shock, posttransplantation tissue rejection, chronic ischemic heart disease, dilated cardiomyopathy, and myocarditis [27–31]. The immunophenotype of this novel hantavirus-induced myocarditis is similar to those described in Coxsackievirus B3–induced myocarditis in genetically immunodeficient mice and in experimental myosin-induced myocarditis. In both conditions, the presence of myocardial diffuse interstitial cellular infiltrate composed of macrophages, T lymphocytes (mainly CD4 cells), and B lymphocytes has been demonstrated [27, 31]. It is conceivable that the pathogenesis of the myocarditis in the acute phase of HPS, similar to the intrinsic mechanism described to occur in the lungs [15], may be caused by early intramyocardial macrophage/monocyte-mediated innate immune responses induced by in situ hantavirus infection followed by migration of T and B lymphocytes from secondary lymphoid organs and reaching the infected myocardium via the bloodstream [15, 22, 32].

Interestingly, interstitial edema was present in HPS myocardium and not in control myocardium. This finding is consistent with the acute phenomenon of hantavirus-induced endothelial cell leakage and the classic HPS-associated severe edema of the lungs, a primary target in the pathogenesis of HPS-associated hantavirus [10, 11, 14, 15]. One could argue that the myocardial interstitial edema might have been altered in heart samples collected between 1 h 40 min and 19 h 45 min after death. Control myocardium from both normal and APALI hearts collected between 2 h 40 min and 28 h after death showed no artifactual separation of myocytes suggestive of interstitial edema.

Cardiac remodeling and TNF-α expression in macrophage-type cells and cardiomyocytes in HPS. Clinical parameters of heart failure, apart from diffuse pulmonary edema, were noted in medical records of 9 patients with HPS who were admitted to the hospital: tachycardia, low blood pressure, renal dysfunction, and hypoxemia. In 2 of these patients, the creatinine kinase–MB fractions in the blood were >25 U/L. Clinical and hemodynamic studies have demonstrated that shock in patients with HPS is characterized by depressed cardiac output, low cardiac stroke volume, hypoxemia, lactic acidosis, high systemic vascular resistance (high afterload), and cardiac dysrhythmias, which contrasts with the high cardiac output and low systemic vascular resistance in sepsis [16, 17, 33–37]. These studies suggest that, at the very least, the cardiovascular events are factors in the high mortality associated with HPS; along with pulmonary edema, these events are considered to be the major distinguishing elements of the disease [7, 10, 11, 14, 16, 17]. Although there is a consensus that the low cardiac output in HPS can be influenced by intravascular volume depletion and pulmonary hypertension, these mechanisms are not sufficient to explain the circulatory shock [16, 17]. A humoral myocardial depressant factor has been suggested to play a role in the reduced cardiac output in HPS shock [17], as has been described in septic states [28, 35, 36]. In the present study, HPS hearts were flabby, showing mild biventricular dilatation and over-stretched myofibers with a markedly decreased minor diameter, possibly reflecting cardiac dysfunction [36, 37]. Studies have shown that activated macrophages and their secreting products can reduce myocyte contractile function [30, 35–37]. Also, expression of TNF-α in the major macrophagic/monocytic cellular component of the hantavirus myocarditis was positively correlated with the number of cells staining positively for IL-10. This finding is consistent with the macrophagic autocrine mechanism of feedback looping between the cytokines IL-10 and TNF-α [14, 36, 38].

TNF-α is a powerful cytokine capable of causing depressed cardiac dysfunction and cardiomyocyte death [35, 36]. Overstretching of myofibers is known to occur after TNF-α exposure; this overstretching can be mediated by sphingosine disruption of calcium transients [36]. Human and experimental studies of viral myocarditis have shown that overexpression of TNF-α causes left-ventricular dysfunction and plays a chief role in myocardial remodeling and heart failure [39–43].

In summary, hantavirus infection induces a typical myocarditis that may be responsible for myocardial depression and shock in fatal HPS. Considering the most recent World Health Organization and International Society and Federation of Cardiology definition of inflammatory cardiomyopathy, as a cardiac disease caused by cellular infiltration (myocarditis) in association with cardiac dysfunction [44], hantavirus myocarditis in the acute phase of HPS can be classified within the group of infectious inflammatory cardiomyopathies and the HPS should be properly named as “hantavirus cardiopulmonary syndrome” (HCPS).

Acknowledgments

We gratefully acknowledge the excellent technical assistance of Ana Maria A. Dorigan, Carla Pagliari, Elaine R. Fernandes, Fernanda Guedes Luiz, Maria Tereza P. Maglia, and Cleusa Fumica H. Takakura.

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