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A Retrospective Analysis of Sera Collected by the Hemorrhagic Fever Commission during the Korean Conflict

James W. LeDuc, Thomas G. Ksiazek,
Cynthia A. Rossi, and Joel M. Dalrymple

From the United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland

More than 600 sera from 245 patients with a clinical diagnosis of hemorrhagic fever were preserved by the Hemorrhagic Fever Commission during the Korean Conflict, 1951-1954. These sera were tested for IgM- and IgG-specific antibodies to Hantaan virus by enzyme immunoassay and for hantaviral antigen by immunoassay; one serum from each patient was tested by plaque reduction neutralization using both Hantaan and Seoul viruses. Only 15 patients failed to develop anti-hantaviral antibodies; most sera contained high titered IgM antibody on admission, and all were IgM-seropositive by day 7 after onset. Attempts to detect hantaviral antigen were unsuccessful. All seropositive patients had highest plaque reduction neutralization titers to Hantaan virus, suggesting that this virus was responsible for the disease seen. These results confirm that hemorrhagic fever of the Korean Conflict was due to Hantaan virus and demonstrate that measurement of specific IgM antibody is the method of choice for diagnosis of acute disease.

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Viruses of the family Bunyaviridae, genus *Hantavirus*, cause a wide range of clinical diseases characterized by fever, renal dysfunction, and frequent hemorrhagic manifestations. Specifically, Hantaan virus is the cause of Korean hemorrhagic fever in Korea and epidemic hemorrhagic fever in China [1, 2]. Puumala virus is the cause of nephropathia epidemica, a related but less severe illness of Scandinavia, western Soviet Union, and Europe [3]. Seoul virus causes a similar disease that is also less severe than that due to Hantaan virus [4, 5]. Recently, another hantavirus, Pirogria virus, was described from the Balkan region of Europe [6]. This virus causes an especially severe disease that appears to result in mortality rates at least as high as those seen with classic Hantaan virus infection. Prospect Hill virus is not known to cause human disease, although antibody has been found among mammalogists in the USA [7, 8]. Hemorrhagic fever with renal syndrome (HFRS) is now used to collectively describe diseases resulting from hantaviral infections.

Before the Korean Conflict HFRS was generally unknown to Western medicine, but it was a significant cause of morbidity and mortality among United Nations forces serving in Korea and rapidly stimulated the formation of the Hemorrhagic Fever Commission to investigate this disease. While the Commission made substantial advances in understanding the clinical disease and improving treatment, the etiologic agent remained elusive. It was only in 1976 that Hantaan vi-

rus, the cause of HFRS, was finally isolated from the lungs of an infected Korean striped field mouse, providing the opportunity for further investigations of this disease [1]. As part of their investigations, the Commission systematically collected and preserved acute and convalescent sera from clinically diagnosed hemorrhagic fever patients. Although we were unable to locate the clinical records for these patients, the serum collections remain intact, and we serologically examined the samples for evidence of hantaviral infection.

Materials and Methods

Serum collection. Sera were obtained from 245 patients with a clinical diagnosis of hemorrhagic fever, representing ~10% of the patients seen during and immediately after the Korean Conflict. Sera were obtained from patients during most months from December 1951 to August 1954 and were labeled with the patient's name and number, date of collection, volume, and a "DD" number, which we believe represents the "day of disease." "Hemorrhagic fever" was typed in red on the top of every sample label. The information on the labels is the only patient information that we have been able to locate, and we have used these dates to calculate days after onset of disease when presenting results. Sera were lyophilized in glass ampules and packed in cardboard boxes in three metal trunks.

Serologic techniques. Sera were rehydrated with sterile, distilled water according to the volume indicated on each label, mixed thoroughly, then transferred to plastic tubes and stored at -30°C until tested. Each sample was tested by enzyme immunoassay for IgM- and IgG-specific anti-Hantaan virus antibodies as follows. Tests for IgM-specific antibody were done by the method of Duermeier et al. [9]. Goat anti-human μ -chain antibody was coated onto polyvinyl chloride (PVC) plates and IgM was captured from a 1:100 or greater dilution of serum. Virus-specific IgM was measured by reacting viral antigen and parallel, mock antigen with the captured IgM. Sera that contained virus-specific IgM captured the viral antigen, which was then measured with an antigen detection system with convalescent sera from rabbits experimentally infected with a Korean

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Reprints or correspondence: Dr. James W. LeDuc, DAD, USAMRIID, Bldg. 1425, Fort Detrick, Frederick, MD 21701-5011.

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hantavirus isolate and horseradish peroxidase-conjugated anti-rabbit antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and an ABTS substrate (Kirkegaard and Perry). Bound enzyme was quantitated at 410 nm and an adjusted optical density (OD) calculated by subtracting the average of the wells containing mock antigen from the virus-specific antigen wells. When this OD exceeded an arbitrary value obtained from the mean of five normal sera plus three standard deviations, sera were titrated by serial twofold dilutions beginning at 1:100; the last dilution exceeding the cutoff value was considered the extinction titer. For tabulating the number and percentage IgM positive by disease day, only sera with titers ≥ 800 were considered positive.

The IgG test was done by the method of Meegan et al. [10]. Hantaan virus was captured on PVC microtiter plates by coating wells with a pool of anti-Hantaan monoclonal antibodies, which acted as an immunocapture antibody, followed by addition of Hantaan viral antigen or similarly prepared mock antigen. Sera were tested in doubling dilutions using wells coated either with Hantaan viral antigen or mock antigen, followed by the addition of horseradish peroxidase-conjugated anti-human IgG (Accurate Chemical and Scientific, Westbury, NY). Bound horseradish peroxidase was measured by using ABTS substrate. The adjusted OD, criteria for positive OD values, and extinction titers were calculated as for IgM antibody.

Attempts were made to measure viral antigen in early acute serum from each patient and from those negative for IgM antibody by adding diluted (1:2 to 1:128) sera to wells coated with Hantaan virus-specific pooled mouse monoclonal antibodies or control fluids: myeloma-induced mouse ascites, followed by rabbit anti-Hantaan-specific antibodies, horseradish peroxidase-conjugated goat anti-rabbit, and ABTS substrate.

To determine the antigenic specificity of the infecting virus, the last convalescent serum from each patient was tested by plaque-reduction neutralization test (PRNT), using both prototype Hantaan virus, strain 76-118, and urban rat-associated Seoul virus, strain 80-39, following procedures described previously [11]. Prior studies demonstrated a difference of ~ 10 -fold or greater between the PRNT titers with homologous and heterologous hantaviruses [6]. Consequently, sera were tested at 1:10 and 1:40 dilutions to both viruses, and the highest convalescent PRNT titer was considered indicative of the infecting virus strain.

Results

More than 600 sera were examined from 245 patients. The modal disease day for the first sample obtained from each patient was day 3, although many sera were drawn on day 2 and a few on day 1. Most patients were again bled ~ 1 week after admission, and a third or additional samples were taken later. Table 1 summarizes the total sera tested by disease day.

Only 15 (6%) of the 245 patients failed to develop anti-hantaviral antibodies over the course of their illness. A few of these sera were from patients with only one available serum sample and could represent patients who died early in disease; however, most were negative in all samples obtained and appear to represent infections with agents other than hantaviruses. Using the worst case analysis, the Commission clinicians were accurate in their clinical diagnosis at least 94%

Table 1. Sera positive for IgM-specific anti-Hantaan virus antibody by disease day for patients serologically confirmed with hemorrhagic fever with renal syndrome.

Disease day	Total positive (≥ 800)/total tested (%)
1	3/5 (60)
2	25/41 (61)
3	47/62 (76)
4	35/40 (88)
5	20/21 (95)
6	14/15 (93)
7	9/9 (100)
8	9/9 (100)
9	1/1 (100)
10	6/6 (100)
11-20	100/100 (100)
21-30	149/149 (100)
31-40	44/44 (100)
41-50	42/42 (100)
≥ 51	30/30 (100)

of the time. Attempts to identify the cause of illness in these 15 non-HFRS cases revealed 1 patient with serologic evidence of leptospirosis; no diagnosis could be made for the other 14.

Seropositive patients developed high-titered IgM anti-Hantaan virus antibody early in the course of their disease (figure 1, table 1). IgM titers were elevated very early, often in the first sample drawn, and quickly reached maximum within the first few days of illness. By comparison, IgG titers rose much more slowly and did not reach maximum until about week 2 of disease.

All attempts to detect hantaviral antigen in samples drawn early in disease or in samples where no IgM antibody was detectable were unsuccessful. All convalescent sera from seropositive patients exhibited the highest PRNT titers to prototype Hantaan virus; titers were either much lower or non-detectable to Seoul virus.

Discussion

The immunoassay results clearly indicate that the hemorrhagic fever seen among United Nations forces during the Korean Conflict was due to infection with a hantavirus. Neutralization test results further suggest that prototype Hantaan virus, rather than urban rat-associated Seoul virus, was the likely infecting agent. While this has been assumed previously, because of the living conditions of deployed troops and intimate association with field rodents, analysis of this very unique serum collection offers the first definitive proof.

Measurement of IgM-specific anti-hantaviral antibody appears to be the method of choice for early diagnosis of HFRS. Specific IgM antibodies were often present in the first serum sample drawn from patients on admission, and all patients had measurable IgM antibodies by day 7 of disease. Further,

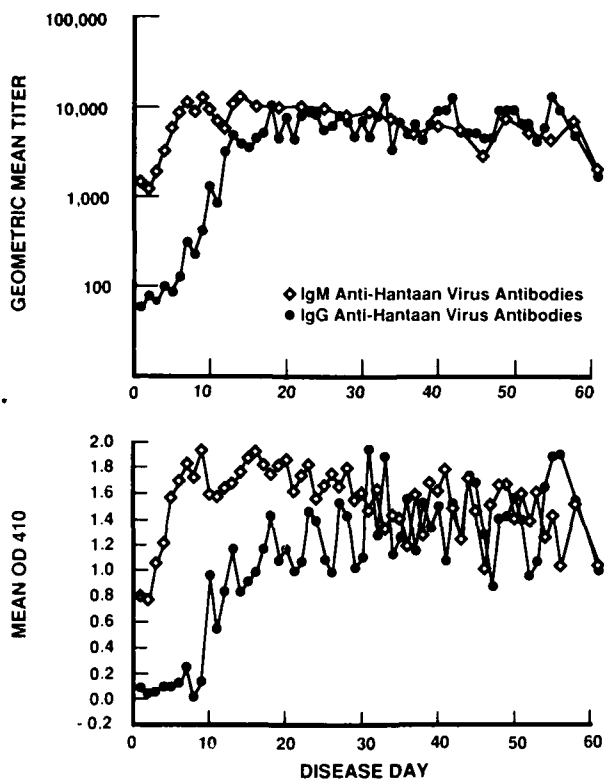


Figure 1. Geometric mean titers and mean optical densities (OD) at 1:100 dilution in enzyme immunoassays for IgM- and IgG-specific anti-Hantaan virus antibodies by disease day among serologically confirmed cases of hemorrhagic fever with renal syndrome.

all attempts to measure hantaviral antigen in sera drawn early in disease, or in samples lacking IgM antibody, were negative. These findings are consistent with our past experience in diagnosing various forms of this disease elsewhere in Asia and Europe and appears to be a general characteristic of the disease (unpublished observations).

In summary, a retrospective serologic analysis of a well-preserved serum collection has incriminated prototype Hantaan virus as the causative agent of hemorrhagic fever in soldiers infected during the Korean Conflict. US physicians were 94% accurate in their clinical diagnosis and, with the excep-

tion of a single case of leptospirosis, the etiology of the other infections remains obscure. Although tests for circulating viral antigen were negative, IgM titers were elevated early in disease, clearly demonstrating their diagnostic potential.

Acknowledgment

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