Detection of Muerto Canyon Virus RNA in Peripheral Blood Mononuclear Cells from Patients with Hantavirus Pulmonary Syndrome

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To determine if Muerto Canyon Virus (MCV) RNA is present in the peripheral blood of patients with hantavirus pulmonary syndrome, a reverse transcriptase-polymerase chain reaction (RT-PCR) assay for MCV RNA was used on blood samples from 20 seropositive case-patients. RNA was prepared from peripheral blood mononuclear cells (PBMC) or blood clot (or both) from 19 and from plasma from 11 case-patients. All 12 blood clot, all 13 PBMC, and 8 of 11 plasma preparations produced an MCV amplification product after RT-PCR with primers to the G2 gene. All of 5 PBMC RNA preparations tested were positive using unnested primers and amplimers, verifying that unique MCV cDNA sequences were amplified. Viral RNA became undetectable in 5 of 7 convalescent samples tested but was present up to day 23 of illness in 2 case-patients.

In the spring of 1993, a cluster of deaths due to an unexplained pulmonary disease superficially resembling adult respiratory distress syndrome was noted in rural residents of the southwestern United States [1-4]. A nonspecific syndrome of myalgias, chills, and fever was followed by the abrupt onset of pulmonary disease. The pulmonary disease stage was manifested clinically by dyspnea, cough, oxygen desaturation, hemodynamic instability, thrombocytopenia, laboratory evidence for coagulopathy, elevated serum lactate dehydrogenase, neutrophilia, and left shift [5]. In the 53 infections known as of January 1994, 32 (60%) resulted in death [6]. The pathologic hallmarks are gross pulmonary...
edema and evidence for massive transudation of proteinaceous fluid into the alveolar air spaces, with little evidence for gross disruption of pulmonary endothelial cells in ultrastructure studies (Feddersen R, personal communication, 1993).

Some features of the disease, such as the thrombocytopenia, coagulopathy, neutropenia, and capillary leak syndrome, were suggestive of certain hemorrhagic fever viruses, such as hantaviruses. A hantavirus was first suspected when IgM and IgG antibodies with cross-reactivity to the known hantaviruses (Seoul virus, Puumala virus, and Hantaan virus) were detected in the serum of case-patients with the pulmonary syndrome [1-3]. Hantaviruses have a minus-stranded RNA genome consisting of the three segments: L, M, and S [7]. A reverse transcriptase–polymerase chain reaction (RT-PCR) assay was developed using primers designed to detect conserved G2 glycoprotein gene sequences of the M segment of Puumala virus and Prospect Hill virus. At autopsy, tissues of case-patients with the new syndrome yielded an amplification product that could be detected by agarose gel electrophoresis. Its sequence was closely related to but as a general means of detecting novel hantaviruses in acute hemorrhagic fever syndromes, usually involving ne-

For one patient, RNA was prepared only from serum. Blood clot RNA was prepared from a total of 12 case-patients, whereas plasma or serum RNA (or both) was prepared from 11 case-patients. PBMC RNA was also prepared from 16 patients admitted with clinical presentations superficially resembling HPS and from 4 normal blood donors. When those 16 patients proved to be seronegative for MCV, and other causes were identified for their illnesses, they were considered to be negative control patients.

RNA was prepared from 5 × 10⁵ PBMC or PMNL, from 200-400 mg of macerated blood clot, or from 100 μL of plasma by acid-phenol extraction in guanidinium isothiocyanate/sarkosyl/ citrate [9]. Serum was used in lieu of plasma when clotted blood was available. After phenol extraction in the presence of 0.2 M sodium acetate (pH 4.0), RNA was precipitated twice in 50% isopropanol and washed twice with 75% ethanol, and the pellet was dried and resuspended in 50 μL of water. One-fifth of the sample volume was subjected to RT-PCR.

RT-PCR with M segment (G2) primers. RT-PCR with nested primers in G2 was done as described [8]. For the first 10 case-patients tested, the outer G2 primer sequences were derived from Puumala and Prospect Hill virus genetic sequences (“consensus primers”). The sense and antisense consensus primers were later found to differ by 4 of 23 nucleotides and 4 of 20 nucleotides from the authentic MCV mRNA or genomic RNA, respectively. The last 10 case-patients were tested with outer primers derived from the sequence of MCV [10] (Genbank accession no. L25783). Those primers had the sequences 5'-AGAAAGATCT-TGTGGGTTTGC-3' (sense) and 5'-CCTGAACCCTCCCGCC- CGT-3' (antisense). Outer primers are spaced 267 nucleotides apart on the MCV M segment. The same inner primers, based on MCV sequences, were used regardless of whether outer primers were consensus or MCV [8]. A 172-bp cDNA amplifier was produced in positive samples and was visualized by agarose gel electrophoresis. Reverse transcription and thermal cycling conditions were similar to those previously described [8], except

**Materials and Methods**

**Case-patients and diagnostic studies.** All but 5 of the 20 hantavirus pulmonary syndrome (HPS) case-patients studied were treated at the University of New Mexico Hospital; the others were treated at other hospitals in New Mexico, Colorado, Arizona, or Montana. The ages of case-patients ranged from 12 to 64 years (mean, 31). There were 10 of each sex; 11 died. Tissues from 10 of those who died were subjected to RT-PCR analysis, and all were found to be positive. All 9 from whom autopsy tissues were available were also found to have viral antigens in pulmonary or renal endothelial cells (or both) by immunoperoxidase studies (Zaki S, personal communication, 1993). All case-patients were seropositive for IgM and IgG antibodies to the MCV nucleocapsid protein (Kužátek T, personal communication, 1994), and all 15 tested had IgG antibodies to the viral G1 glycoprotein [15]. Ten HPS case-patients were treated with ribavirin.

**Blood samples and RNA preparation.** Acute-phase blood samples from all HPS case-patients were available in very limited amounts. All case-patients considered to be in the acute phase of HPS were tested within the first 6 days of hospitalization. Anticoagulated blood was available from 13 of the case-patients. It was processed into PBMC and plasma fractions by ficoll-hypaque density gradient centrifugation (Histopaque 1077: Sigma, St. Louis) according to the manufacturer’s instructions. In three instances, plasma, PBMC, and polymorphonuclear leukocytes (PMNL) were prepared from a single blood sample using a three-density gradient of blood layered on Histopaque 1077 and Histopaque 1119. Purified cells were pelleted twice in sterile PBS. In most cases, RNA was prepared immediately from the pelleted cells, but cells cryopreserved in 10% dimethyl sulfoxide in tissue culture medium also were usable in PCR studies.

RNA from 5 case-patients was prepared only from blood clots. For one patient, RNA was prepared only from serum. Blood clot RNA was prepared from a total of 12 case-patients, whereas plasma or serum RNA (or both) was prepared from 11 case-patients. PBMC RNA was also prepared from 16 patients admitted with clinical presentations superficially resembling HPS and from 4 normal blood donors. When those 16 patients proved to be seronegative for MCV, and other causes were identified for their illnesses, they were considered to be negative control patients.

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**RT-PCR with M segment (G1) primers.** RT-PCR with nested primers in G1 was done as described [8]. For the first 10 case-patients tested, the outer G1 primer sequences were derived from Puumala and Prospect Hill virus genetic sequences (“consensus primers”). The sense and antisense consensus primers were later found to differ by 4 of 23 nucleotides and 4 of 20 nucleotides from the authentic MCV mRNA or genomic RNA, respectively. The last 10 case-patients were tested with outer primers derived from the sequence of MCV [10] (Genbank accession no. L25783). Those primers had the sequences 5'-AGAAAGATCT-TGTGGGTTTGC-3' (sense) and 5'-CCTGAACCCTCCCGCC-CGT-3' (antisense). Outer primers are spaced 267 nucleotides apart on the MCV M segment. The same inner primers, based on MCV sequences, were used regardless of whether outer primers were consensus or MCV [8]. A 172-bp cDNA amplifier was produced in positive samples and was visualized by agarose gel electrophoresis. Reverse transcription and thermal cycling conditions were similar to those previously described [8], except
that a higher PCR annealing temperature (48°C) was used when the MCV outer primers were used.

**S segment RT-PCR.** Unnested primers were designed from the 3H226 S segment of MCV [9] (Genbank accession no. U02474). They were spaced 256 nucleotides apart on the segment. The sense-strand primer had the sequence 5'-AGCACATTCAGACGACGCGGC-3', and the antisense primer 5'-GGATAAATCGGTAATGCAAAACT-3'. Reverse transcription was done for 1 h at 42°C, followed by thermal cycling at 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min for 50 cycles.

DNA sequencing. To verify that a given amplimer was a unique product of MCV, five 172-bp G2 amplimers and four 256-bp S segment amplimers were cloned into the plasmid vector pCRII according to the manufacturer's instructions (Invitrogen, San Diego). The nucleotide sequence was determined by the dideoxy method according to the instructions for Sequenase (US Biochemicals, Cleveland). G2 amplimers from 15 case-patients were sequenced directly without cloning, as described [8, 10]; 4 of those 15 were also sequenced from cloned DNA.

**Results**

**HPS is associated with circulating viral RNA in plasma and PBMC.** Table 1 summarizes the G2 gene RT-PCR results obtained from various blood fractions in case-patients admitted with HPS. Our sample supply was limited to some extent by the availability of blood samples in certain case-patients in severe respiratory distress. Of the blood fractions examined, PBMC and blood clots appeared to be optimal, since all 13 PBMC samples and all 12 blood clot samples contained detectable viral RNA. RNA prepared from plasma or serum was positive in 8 of 11 case-patients. Four of the 5 serum samples were positive, compared with 6 of the 9 plasma samples. Two of 3 PMNL samples were also positive, but 1 of the positive samples produced a rather weak signal compared with a side-by-side analysis of PBMC from the same patient (data not shown). In all negative assays using plasma or PMNL templates, consensus outer primers were used in nested RT-PCR reactions rather than primers based on MCV sequences. We have not evaluated whether those samples would still be negative using primers based on MCV sequences.

**Amplified material from HPS case-patients is derived from the MCV genome.** The nested G2 gene PCR primer system produced a product of the predicted 172 bp that was easily visible on agarose gel electrophoresis (figure 1). No product of similar size could be detected after RT-PCR was done on samples from 16 control patients with various pulmonary diseases who were seronegative for MCV. Four RNA samples from normal blood donors were also negative. The nucleotide sequence of the 172-bp G2 amplimer was determined in 16 patient samples. With the exception of two pairs of samples in which two sequences were identical, each amplimer was different from any other amplimer. Samples with identical sequences had been processed at different times, ruling out cross-contamination during sample preparation. As described previously, each amplimer had the sequence predicted for MCV [8]. Individual sequences varied from one another by 0–15 %. Nearly all variations were synonymous changes.

The outer primers derived from MCV sequences tended to result in a more intense band than did the Puumala and Prospect Hill virus consensus primers, even though the same inner primers were used. However, there was no systematic difference in sensitivity, since MCV G2 RNA could be detected in PBMC or blood clots from all case-patients with HPS who were tested with either set of outer primers.

The PBMC RNA samples from 5 HPS case-patients were also subjected to RT-PCR with the unnested primer system in the gene for the nucleocapsid protein N. The predicted 256-bp cDNA amplification product was visible on agarose gel electrophoresis from all 5 of those samples but was not evident after RT-PCR of 4 control RNA samples. Four of the five bands were cloned and the nucleotide sequence determined. Variation of 2.5%–10.9% was noted among the four clones. Only synonymous changes were detected.

**Viral RNA is absent in the PBMC of most case-patients in convalescence.** On six occasions we were able to obtain a second (convalescent) blood sample from 5 case-patients whose PBMC contained detectable viral RNA during their acute illness. Those samples were obtained at days 19 and 123 (relative to hospital admission) from patient 1B, day 23 from patient 2J, day 22 from patient 1H, day 38 from patient 2R, and day 162 from patient 3M. A seventh sample was obtained from patient 2H, who was recognized in November 1993 to have had HPS in April 1993. His diagnosis was based on a characteristic clinical course and the presence of IgG and IgM antibodies to the MCV nucleocapsid protein and IgG antibodies to the GI glycoprotein [15]. A convalescent blood sample was obtained on day 269 after his hospital

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**Table 1.** Reverse transcription–polymerase chain reaction assay using nested primers in G2 on various blood fractions from patients with hantavirus pulmonary syndrome.

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Plasma</th>
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<th>PMNL</th>
<th>Clot</th>
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NOTE. PBMC: peripheral blood mononuclear cells; PMNL: polymorphonuclear leukocytes; Pos: band present on ethidium bromide gel; Neg. no band present; NT: not tested.
analysis of peripheral blood mononuclear cell RNA from 3 patients on admission, but no PBMC or blood clot sample was available from the acute phase of his illness.

On one of two RT-PCR studies done on the day 19 sample from patient 1B, a faint band of the appropriate size was visible after amplification with the nested G2 primers (data not shown). A second assay of that RNA sample was negative. A blood clot sample from patient 2J was positive at day 23. With those exceptions, all convalescent blood samples were negative for MCV RNA. Four examples of convalescent PBMC RNA samples that were negative by RT-PCR are shown in figure 1.

Most RT-PCR studies done on patients who received ribavirin used blood samples drawn within the first 4 days of ribavirin therapy. However, several patients were examined after >4 days of therapy and remained positive for MCV RNA. Two blood clot samples were obtained as late as day 10 or day 22 of ribavirin therapy in case-patients 3E and 2J, respectively; both were positive.

Bronchoalveolar lavage samples. RNA preparations were prepared from the cellular fraction of 50 mL of excess bronchoalveolar lavage fluid from 3 case-patients. Those lavage samples represented ~90% of the return obtained from 125 mL of saline. Another preparation was made from 100 μL of the supernatant fraction from 1. In all cases, a PBMC RNA preparation was made on the same day and was positive by RT-PCR. Three of the 4 bronchoalveolar lavage RNA preparations were negative. The cellular fraction of lavage fluid from 1 case-patient was positive.

MCV viral RNA is detectable in blood plasma, serum, and cells. MCV RNA was detectable in most (8/11 [73%]) plasma or serum samples from case-patients in the acute phase of HPS. Viral RNA was detectable for as long as 9 days after the onset of illness, but only a single plasma sample was evaluated after 9 days. That sample was negative at day 22 relative to onset of illness or day 19 relative to hospitalization (data not shown).

Discussion

The high fraction of positive assays using serum or plasma template supports the concept that patients are viremic in the acute phase of HPS. Although serum or plasma samples were positive at about the same frequency, we do not believe that our study demonstrates conclusively that our acute HPS case-patients were viremic. Viral RNA could be present at low levels in contaminating cellular debris or platelets.

The consistent presence of detectable MCV RNA in circulating mononuclear cells has implications for the diagnosis, treatment, pathogenesis, and possibly transmission of MCV. For diagnostic studies, RT-PCR may play a role as a reference standard in the evaluation of the efficacy of other test formats. If it becomes possible to develop quantitative RT-PCR systems for MCV, it may play a role in monitoring the effect of antiviral or other therapies on virus load. Ribavirin has been in an unblinded trial for treatment of HPS since June 1993. In a small number of patients examined after >4 days of ribavirin therapy, viral RNA remained detectable. Two HPS case-patients we studied remained positive for MCV RNA after 10 days or 21 days of ribavirin therapy.

The persistence of viral RNA in acute HPS and in nephropathia epidemica [14] can be interpreted as evidence that the continued presence of virus may play a role in the expression of acute hantavirus disease. At the time of the positive PCR analyses, all 15 case-patients studied already had detectable IgG antibodies to MCV antigens (Ksiazek T, personal communication) [15]. Finally, the demonstration of viral genetic material in case-patients in the acute stage of HPS suggests that blood samples from such patients should continue to be handled with caution, with particular attention to avoiding the generation of aerosols. However, there have been no reports of transmission of hantaviruses in persons handling clinical samples from HPS or hemorrhagic fever with renal syndrome patients. We did not examine the infectiousness of our patient blood samples in vitro or in animals because of the great difficulty of primary isolation of hantaviruses.

At autopsy, viral RNA can be detected in homogenates of most organs examined in HPS case-patients [8]. This study supports the concept that MCV becomes widely disseminated in the course of acute HPS. Previous studies, using immunologic detection of cell-associated hantavirus anti-
hantavirus antigens were detectable primarily in monocytes and B lymphocytes [16]. Attempts to detect MCV antigens in ex vivo PBMC preparations, using a monoclonal antibody that recognizes the MCV nucleocapsid protein [17] (Zaki S, personal communication, 1993) have been inconclusive (Mills R, Young S, unpublished data, 1993). Thus, we are unable to determine precisely which cells in the PBMC fraction contain viral antigens or RNA.

Only one of three cell pellets from bronchoalveolar lavage specimens was positive for viral RNA. The lavage specimens from HPS patients generally have high protein concentration but are low in cellularity (Simpson S, personal communication). Cell quantitation was not available for our bronchoalveolar lavage specimens, but the single MCV RNA–positive sample had a much larger cell pellet than the 2 negative samples. Immunohistochemical studies by Zaki et al. [17] indicate that the endothelial cell is the predominant tissue target for infection by MCV. It is unlikely that many endothelial cells are extracted during bronchoalveolar lavage. We believe the low rate of successful amplification of MCV RNA from bronchoalveolar lavage specimens could be due to the low cell numbers we obtained in our samples.

RT-PCR is an exceptionally sensitive method for detection of hantaviruses in acute infection. In combination with DNA sequencing, it offers a level of diagnostic specificity that is not possible with immunologic methods for detecting viral antigens. For hantaviruses, it offers far greater speed and sensitivity than does virus isolation. However, a positive RT-PCR result does not necessarily mean that a specimen contains infectious virus, and determining with precision which cell type contains viral RNA by RT-PCR is difficult.

Rodents infected with hantaviruses develop chronic viremia and viruria [13]. Although this has not yet been demonstrated to occur after human infection by other hantaviruses, the possibility of chronic human infection has not been extensively studied. Our studies with a limited number of patients suggest that human infection by MCV is transient. This matter should be examined in a larger number of patients.

Acknowledgments

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References

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References