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Short Communication

Prompt Administration of Crimean-Congo Hemorrhagic Fever (CCHF) Virus Hyperimmunoglobulin in Patients Diagnosed with CCHF and Viral Load Monitorization by Reverse Transcriptase-PCR

Ayhan Kubar*, Mustafa Haciomeroglu[1], Aykut Ozkul[2], Umit Bagriacik[3], Esragul Akinci[4], Kenan Sener, and Hurrem Bodur[4]

Gulhane Military School of Medicine, Ankara; [1]Refik Saydam Hygiene Center, Ankara; [2]Ankara University, Ankara; [3]Gazi University, Ankara; and [4]Ankara Numune Training and Research Hospital, Ankara, Turkey

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SUMMARY: Crimean-Congo hemorrhagic fever virus (CCHFV), a member of the genus *Nairovirus* of the family *Bunyaviridae*, causes a severe disease in humans with high mortality rates. In Turkey, the number of patients with CCHF has increased since 2002. Here, we aimed to treat CCHF patients with CCHFV hyperimmunoglobulin. We prepared a CCHFV hyperimmunoglobulin product from 22 individuals who survived CCHF infection. A total of 26 CCHF patients were enrolled into this study. For CCHFV hyperimmunoglobulin administration, a Kubar Unit (KU) was defined. As a standard therapeutic approach, 400 KU of hyperimmunoglobulin were given to each patient as a single dose before viral load was detected. We used one-step real-time reverse transcriptase-PCR to monitor the viral load of CCHF patients. According to the one-step real-time PCR results, 15 patients with a viral load of 10^8 copies/mL or more were defined as high risk. In this high-risk group, the survival rate was found to be 86.6% (13/15) and 2 patients died despite CCHFV hyperimmunoglobulin administration. CCHF is a very serious and highly fatal infection, particularly for patients in the defined high-risk group. Prompt administration of CCHFV hyperimmunoglobulin might be a very promising new treatment approach, especially for high-risk individuals.

Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne disease named for the causative agent, Crimean-Congo hemorrhagic fever virus (CCHFV), which is a member of the genus *Nairovirus* (family *Bunyaviridae*) (1). The viral genome has the characteristic features of other family members, and is composed of three negative-strand RNA segments, S, M, and L, which encode the N nucleocapsid, the Gn and Gc glycoproteins, and the L polymerase, respectively (2,3). CCHFV glycoprotein biogenesis is considerably more complex than that of viruses in the other genera of the family *Bunyaviridae* (4). CCHF has come to the attention of the medical community due to its high mortality rate (5–30%) (1). In Turkey, by the end of 2009, there were 1,318 confirmed cases and 63 deaths (a case-fatality rate of 4.8%) (5). In general, humans are incidental, ''dead-end,'' hosts for CCHFV. The pathogenesis of CCHF appears to be quite complex. Furthermore, it has not been well characterized, and many questions remain to be answered (6). The disease can be divided into different clinical stages, and its rapid progression may be due to yet unknown reasons (7). An understanding of the dynamics of CCHFV infection and its treatment modalities would have a significant impact on the management, particularly for patients with poor prognosis by definition. A few recent studies have reported that the most important indicator for clinical status and fatality was the patient's serum viral load (8,9). If a patient's viral load was less than 10^8 or 10^9 copies/mL, the disease usually did not progress to a fatal outcome. In contrast, patients with a viral load greater than or equal to 10^8 or 10^9 copies/mL had a poor prognosis (8,9).

There is no specific therapy for CCHF. Although ribavirin has been the most commonly applied therapy in clinical practice, the effect of ribavirin therapy on the outcome of CCHF is controversial (1,10,11), which emphasizes the need for novel approaches.

In this study, we collected plasma from 22 healthy donors who were residents of CCHF endemic regions in Turkey, and prepared CCHFV hyperimmunoglobulin from this pooled plasma. All the people who gave plasma were in good health and met blood donation criteria, (i.e., negative tests for hepatitis B virus [HBV], hepatitis C virus [HCV], and human immunodeficiency virus [HIV]).

The final product, CCHFV-specific hyperimmunoglobulin, was obtained after a combination of ammonium sulfate precipitation and ion-exchange chromatography, and was prepared in 5-mL aliquots. All processes related to purification and packing were performed under Good Manufacturing Practice (GMP) conditions.

To test the specificity of the prepared CCHFV immunoglobulin for the CCHFV Ank-1 strain, we purified

*Corresponding author: Mailing address: Gulhane Military School of Medicine, 06018 Ankara, Turkey. Tel: +90 312 3043414, Fax: +90 312 3043402, E-mail: akubar@hotmail.com

the Gn and Gc glycoproteins of CCHFV by isoelectric focusing. For this purpose, the CCHFV-Ank-1 strain was propagated in Vero E6 cells and then harvested and inactivated with polyimin (12,13). The final virus suspension was mixed with 2% of a 2–11 ampholyte solution (Servalyte; Serva, Munchen, Germany) in order to obtain a pH gradient in a Rotofor cell (Bio-Rad, Hercules, Calif., USA). Isoelectric focusing in the Rotofor cell was continued for 4 h. The pH of each fraction was measured, and was then analyzed by SDS-PAGE for Western blotting. In fractions containing the Gn and Gc glycoproteins, the pH values ranged from 3.5 to 4.5. The isoelectric point was pH 3.5 for lane 1, and 4.5 for lane 6 (Fig. 1). While only a single band (a 64-kDa glycoprotein dimer), was observed in lanes 1–5 despite different pH conditions (pH range 3.5–4.3), 3 bands (37-, 64-, and 78-kDa glycoproteins) was observed in lane 6 at pH 4.5. The specificity of the prepared CCHFV immunoglobulin was shown by Western blotting as strongly reactive (high density) bands between the purified Gn and Gc glycoproteins and the hyperimmunoglobulin product we produced.

From both our clinical and laboratory experience, the highest viral load detected in fatal cases was 10^{12} copies/mL (unpublished observation). The amount of CCHFV hyperimmunoglobulin administered was based on the neutralization power, i.e., the amount that could achieve 100% inhibition of the highest viral load experienced in fatal cases. The amount of antibody required to neutralize 10 mL of patient plasma that may contain up to 10^{12} copies/mL of virus was defined as 1 Kubar Unit (KU). Since 10 mL of patient plasma contained a total of 10^{13} copies of the virus, by definition, 1

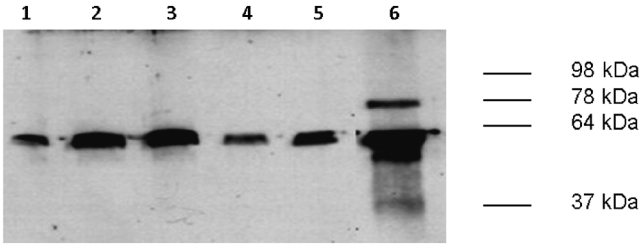

Fig. 1. Western blot analysis of the CCHFV Gn and Gc proteins. While lanes 1–5 had mainly dimeric 64-kDa CCHFV Gn proteins that were harvested in 5 different tubes at different isoelectric focusing points, lane 6 had 37-kDa monomeric and 64-kDa dimeric Gn proteins as well as a 78-kDa Gc proteins. Note that the Gn and Gc bands that reacted with the purified hyperimmunoglobulin are very dense.

Table 1. Viral load data of the patients in high-risk group having viral loads of 10^8 copies/mL or higher

Case no. sex, age	C_T values[1] at all time intervals and viral loads at 0 and 48 h										
	0 h	3 h	6 h	12 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
1 M, 28	17.7 2.6×10^{10}	No data	20.1	23.0	25.6	26.7 6.2×10^6	29.1	UT[2]			
2 M, 60	20.5 5.3×10^9	20.2	20.6	20.9	21.3	22.7 2.8×10^8	31.5	UT			
3 M, 25	21.0 7.9×10^8	20.8	21.6	21.3	22.0	25.3 7.9×10^6	26.2	27.8	31.6	UT	
4 F, 57	20.3 6.0×10^9	20.9	20.8	19.9	21.8	25.9 8.8×10^6	29.3	20.7	31.9	30.3	UT
7[3] M, 53	21.9 5.2×10^8	21.2	22.1	22.6	19.6	19.2 7.1×10^9	19.1				
8 M, 53	20.0 2.5×10^9	20.2	21.1	19.9	23.5	24.9 4.7×10^7	25.2	25.4	30.1	UT	
9 M, 21	21.0 7.7×10^8	21.9	24.8	23.7	21.3	23.8 7.2×10^7	28.6	33.1	UT		
10 F, 64	23.0 1.5×10^8	24.9	26.0	25.3	25.8	29.2 7.5×10^4	37.3	UT			
12 F, 47	22.6 2.9×10^8	22.5	22.8	22.9	22.4	25.2 7.8×10^6	26.5	32.4	UT		
13 M, 49	16.0 3.3×10^{11}	16.7	16.5	16.2	17.4	17.5 4.7×10^{10}	19.5	28.8	36.1	UT	
16 M, 73	22.0 5.1×10^8	22.2	22.3	23.7	23.4	29.7 7.2×10^4	UT				
17 M, 76	22.5 3.4×10^8	24.3	27.9	27.4	27.0	35.5 1.0×10^3	UT				
18[3] M, 59	20.2 2.3×10^9	20.8	20.4	19.6	23.4	17.8 2.7×10^{10}					
19 F, 49	18.8 8.7×10^9	21.4	21.0	20.9	23.1	23.9 4.6×10^7	24.0	30.8	UT		
24 M, 21	22.8 2.3×10^8	21.8	23.0	22.0	23.8	27.0 2.9×10^6	34.5	UT			

[1]: Higher CT values correlate with low viral loads and vice versa.
[2]: UT, under threshold value.
[3]: Patients died.

KU is the amount of CCHFV hyperimmunoglobulin that can neutralize 10^{13} copies/mL of CCHFV Ank-1 strain in vitro. In an in vitro plaque reduction neutralization assay, we determined that a 1:40 dilution of the purified hyperimmunoglobulin was capable of completely inhibiting the growth of CCHFV-Ank-1 strain RNA titer of 10^{13} copies/mL (14). In other words, 1 mL of CCHFV hyperimmunoglobulin contained 40 KU, and each 5 mL vial had a potency of 200 KU. Finally, considering the definition of a KU and the predicted mean normal plasma volume in adults, nearly 400 KU of CCHFV hyperimmunoglobulin were required for a single dose in each patient (15).

A total of 26 patients admitted to Ankara Numune Training and Research Hospital who were tentatively diagnosed with CCHF according to their clinical histories and/or clinical characteristics were enrolled in this study. When the patients were admitted to the clinic, (defined as zero time), 10 mL of CCHFV hyperimmunoglobulin was promptly administrated by intravenous route (IV) in a single dose after the first blood sampling. To monitor viral load, blood samples were taken at 3, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h of hospitalization. One-step real-time reverse transcriptase (RT)-PCR (7500 real time PCR system; Applied Biosystem, Foster City, Calif., USA) was used to monitor the viral load, technique that we first developed (14).

On first admission, signed consent forms were obtained from all patients or relatives. This study was approved by the Central Ethics Committee in Ankara, and the relevant reference number for the judgment is 049479.

The viral loads detected at different time points were expressed as both copies/mL and cycle threshold (C_T) values. The viral loads were expressed as C_T values at all time periods; however, because of the confusing nature of exponential numbers and for ease of comparison, the copies/mL data are only shown for the zero time and 48 h detections (Tables 1 and 2). In duplicate runs of one-step real-time RT-PCR analysis, there were no two reciprocal tests in which the results exceeded the 0.5 C_T value.

It is well known that infection due to the CCHFV has a high mortality rate that varies from 5% to 30%, and a specific therapy is not yet available (1,10,11). Although it seems that CCHF cases in Turkey are less severe and the case-fatality rate is relatively lower, understanding the dynamics of infection and treatment modalities would have a significant impact on the management of high-risk patients with poor prognosis. The estimated dose seemed to work well in all patients, especially those in the high-risk group. The high affinity of purified hyperimmunoglobulin for the Gn and Gc proteins, as shown by Western-blot analyses, likely plays a significant role in the success of the therapy due to its neutralizing activity (Fig. 1).

Although the use of passive immunotherapy for the management of CCHF disease was first reported in 1990, this approach has not been widely appreciated by the experts until now (16). In the study, the authors prepared specific immunoglobulin from the plasma of donors who had been immunized with a single dose of CCHF vaccine. Although in this earlier study, the authors determined a titer for the antibodies against CCHFV by immunodiffusion, they did not categorize the patients by disease severity using a measurable method. Such discrimination would have allowed the authors to evaluate which patients could really benefit from the passive immunotherapy.

Table 2. Viral load data of the patients in low-risk group having viral load of 10^7 copies/mL or lower

Case no. sex, age	C_T values[1] at all time intervals and viral loads at 0 h						
	0 h	3 h	6 h	12 h	24 h	48 h	72 h
5 F, 58	27.3 3.2×10^6	27.6	31.6	UT[2]			
6 F, 39	24.9 4.6×10^7	26.9	27.2	30.9	UT		
11 F, 58	23.5 7.9×10^7	24.3	25.9	23.4	25.2	32.5	UT
14 F, 41	24.6 5.2×10^7	25.0	25.1	31.2	UT		
15 F, 66	27.6 2.2×10^6	29.7	36.1	28.3	30.3	UT	
20 F, 63	28.5 3.0×10^5	30.5	29.7	30.9	31.2	UT	
21 F, 54	27.1 4.1×10^6	31.5	28.0	30.6	31.1	33.2	UT
22 F, 53	27.3 3.3×10^6	28.6	28.1	28.3	29.9	33.3	UT
23 M, 62	25.9 8.9×10^6	26.0	27.2	28.4	32.6	UT	
25 F, 35	29.5 7.0×10^4	31.2	30.9	30.8	35.0	UT	
26 M, 61	28.3 3.4×10^5	26.0	27.5	25.1	33.1	UT	

[1]: Higher CT values correlate with low viral loads and vice versa.
[2]: UT, under threshold value.

Some very recent studies have reported a direct relationship between viral load and mortality rate (8,9). Although there are a very limited number of studies on this subject, a viral load of at least 10^8 copies/mL appear to be a critical level for the estimation of prognosis in these patients. In light of this, after we obtained PCR test results for our patients, it was possible to divide the patients into high- and low-risk groups according to their viral load. Of our 26 patients, 11 had a low viral load of 10^7 copies/mL or lower (Table 2). The patients in the low-risk group were expected to survive, even if no antiviral therapeutic intervention was applied. Therefore, we thought that the success rate of our treatment approach should be evaluated only in the high-risk group (Table 1).

As shown in Table 3, it is noteworthy that except the viral load data, the basic laboratory studies and clinical characteristics of the patients were not very helpful in determining the severity of the disease. For example, while the platelet count in 2 patients who died was in the range of 20,000–23,000/mm^3, it was in the range of 10,000–18,000/mm^3 in 8 survived patients. Furthermore, the presence of anti-CCHFV antibodies (IgM and/or IgG) in our patients at the early stage of infection might not mean that these were completely protective, unless the neutralizing antibody content was at an optimal level.

The soluble glycoproteins and mucin-like proteins of

Table 3. Overall selected laboratory and clinical characteristics of CCHF patients in the study

Case no./ risk group	Selected laboratory parameters								Selected clinical characteristics		
	Anti-CCHF IgM/IgG	Leukocyte count[1] (cells/mm^3)	Platelet count[1] (platelets/mm^3)	AST[2] (U/L)	ALT[2] (U/L)	LDH[2] (U/L)	CPK[2] (U/L)	INR[2]	Fever	Bleeding (any kind)	Day of symptom before admission
1/HR	+/−	2,600	10,000	697	303	3,151	5,957	1.29	+	+	3
2/HR	+/+	1,400	15,000	542	876	939	311	0.98	+	+	10
3/HR	+/−	1,600	90,000	36	21	239	81	1.26	+	−	1
4/HR	+/+	1,300	10,000	1,319	464	1,825	686	1.15	+	+	7
5/LR	+/−	1,500	75,000	699	358	740	298	1.06	+	+	5
6/LR	+/−	2,100	81,000	141	102	340	293	1.28	+	+	5
7/HR[3]	+/−	6,900	23,000	1,929	721	5,342	3,452	1.42	+	+	6
8/HR	+/+	1,100	12,000	340	197	943	1,778	1.47	+	−	1
9/HR	+/−	1,400	69,000	170	69	365	894	1.31	+	−	2
10/HR	+/−	2,200	18,000	374	154	776	351	1.00	+	+	1
11/LR	+/−	1,300	73,000	44	37	257	50	1.11	+	−	3
12/HR	+/−	1,900	118,000	249	160	393	177	1.11	+	−	2
13/HR	+/+	2,700	10,000	205	144	522	185	1.30	+	−	1
14/LR	+/−	1,400	56,000	116	125	280	97	1.00	+	−	1
15/LR	+/−	1,200	59,000	149	76	421	210	1.08	+	+	1
16/HR	+/−	1,900	69,000	90	46	302	714	1.05	+	−	2
17/HR	+/−	2,000	83,000	71	59	241	194	1.25	+	+	1
18/HR[3]	+/−	1,500	20,000	501	208	1,431	2,227	1.36	+	+	4
19/HR	+/+	500	57,000	152	84	292	167	1.14	+	+	1
20/LR	+/−	2,400	25,000	573	367	743	759	1.27	+	+	4
21/LR	+/−	2,000	17,000	323	76	531	849	1.00	+	−	2
22/LR	+/−	2,300	114,000	107	91	294	131	1.12	+	+	2
23/LR	+/−	6,300	40,000	101	51	288	135	1.00	+	−	1
24/HR	+/+	2,100	16,000	327	120	667	876	1.20	+	−	1
25/LR	+/−	1,900	58,000	117	63	222	49	1.08	−	−	1
26/LR	+/−	3,200	29,000	396	239	556	907	1.06	+	+	1

[1]: Lowest values during the course.
[2]: Highest values during the course.
[3]: Patients died.
AST, aspartate transaminase; ALT, alanine transaminase; LDH, lactate dehydrogenase; CPK, creatine phosphokinase; INR, international normalized ratio; HR, high risk; LR, low risk.

CCHFV are secreted to the medium in significant quantities, and these proteins could play an important role in viral pathogenesis (17,18). Therefore, our basic goal of administering CCHFV hyperimmunoglobulin as a prompt therapeutic intervention was to stop the new attachment of viral particles to uninfected cells and to block these secreted or scattered proteins. This approach is especially valuable for achieving a comparably high survival rate for patients in the high-risk group. In previous studies, the fatality rate was found to be as high as 90% in the high-risk group in the absence of antiviral intervention (8,9). In the present study, the fatality rate was found to be 13.3% (2/15) in the high-risk group when CCHFV hyperimmunoglobulin was administered. Of these 15 high-risk patients, 2 (Cases 7 and 18) died at 72 and 48 h, respectively, despite the treatment. In the last PCR test prior to death, their C_T values were lower than those at zero time, and their corresponding viral loads were 10^9 and 10^{10} copies/mL, respectively (Table 1). Interestingly, when the viral load values (copies/mL) at both the zero and 48-h time points for all patients in high-risk group were carefully evaluated (Table 1), it could be concluded that a patient would survive if his/her 48-h viral load value was at least 1 log lower than that at zero time. In contrast, a 1-log increase could be a possible indicator for poor prognosis or fatality.

For the two fatal cases in the high-risk group, the increased viral loads despite the therapy suggest that the viral strain RNAs in these patients might have some mutations (mainly in the M segment) that caused a change in the protein structure targeted by the CCHFV hyperimmunoglobulin (19). If the strain variation is significant, collecting convalescent plasma from many more people representing a wider endemic area and using more than one CCHFV strain in the plaque neutralization assays would likely be the best approaches for overcoming this problem.

One of the most striking findings in our study was the survival of Case 13 who had a viral load of 10^{11} copies/mL. In our previous routine clinical/laboratory observations, we had not experienced any patient that survived with a viral load of 10^{11} copies/mL. This finding might indicate that the antigens of the infecting viral strain and the neutralizing antibodies in CCHFV hyperimmunoglobulin were compatible.

We had a noteworthy observation in the viral load of Case 4. Up to 72 h, we detected a declining viral load, and then, at 96 h, we detected an immediate increase to nearly that at zero time. At 120 h, viral load decreased

again (Table 1). We called this trend, a steadily declining viral load that then instantly increased to the zero time value, the ''shuttle phenomenon.'' During this period, no discernible change in clinical status was observed in this patient. We thought that the shuttle phenomenon might be related to viral reentry due to the lysis of infected cells especially from the liver. As a result, it could be speculated that viral propagation during the shuttle phenomenon was successfully neutralized by the CCHFV hyperimmunoglobulin. In turn, a sudden 5-log decrease in viral load (from 10^9 to 10^4) over the next 24 h could be explained by the rapid removal of hyperimmunoglobulin-opsonized CCHFV by leukocytes.

In conclusion, CCHF is a very serious and highly fatal infection, especially for patients in the high-risk group who have the viral loads greater than or equal to 10^8 copies/mL. CCHFV hyperimmunoglobulin administration appears to be a very promising approach for treating CCHF, especially for high-risk patients.

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Conflict of interest None to declare.

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