Interacting Roles of Immune Mechanisms and Viral Load in the Pathogenesis of Crimean-Congo Hemorrhagic Fever

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Until now, the pathogenesis of Crimean-Congo hemorrhagic fever (CCHF) has not been well described. However, it has been hypothesized that it could be a result of the direct injury of virus-infected tissues in combination with the indirect effects of host immune responses, including cytokines. To shed more light on the role of viral load and cytokines, differential influences of CCHF virus (CCHFV) RNA load, antibody response, and cytokine production on severity and outcome of the disease were studied in sera of 46 patients with confirmed acute CCHF from Kosovo. In this study, viral load proved to be strongly related to the severity and outcome of the disease, with higher viral loads detected in patients with fatal outcomes than in surviving patients. Additionally, patients with fatal outcome had on average a weaker antibody response, if one was present at all. High levels of interleukin-10 (IL-10), gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) were associated with poor outcome, since detected concentrations were highest in patients with fatal outcome and lowest in patients with moderate disease course. Interestingly, reduced levels of IL-12 were detected in all CCHF patients. Our study favors the hypothesis that CCHF could be a result of a delayed and downregulated immune response caused by IL-10, which leads to an increased replication and spread of CCHFV throughout the body. This consequently triggers increased production of IFN-γ and TNF-α, cytokines mediating vascular dysfunction, disseminated intravascular coagulation, organ failure, and shock.

A common thread among viral hemorrhagic fevers (VHF) is the ability of the etiologic agent to disable the host immune response by targeting and manipulating the cells that initiate the antiviral response, leading to overwhelming viral burdens and immune and vascular dysregulation. The endothelial damage observed could therefore be a result of the direct effects of virus replication and/or the indirect result of multiple host-induced mechanisms, including cytokines (13, 20, 35).

Crimean-Congo hemorrhagic fever (CCHF) is a potentially fatal tick-borne viral zoonosis (mortality rates are as high as 30%), with reported cases in parts of Africa, Asia, Eastern Europe, and the Middle East. The causative agent, CCHF virus (CCHFV), a member of the genus Nairovirus, family Bunyaviridae, is the most extensively spread tick-borne virus and the second most widespread of all medically important arboviruses, after dengue virus (6, 22, 42). As with other vector-borne diseases, the distribution of disease closely follows the global distribution of its vector, Hyalomma sp. ticks. Human beings become infected through tick bites or by contact with blood, blood-containing body fluids, or tissues of viremic livestock or human patients during the acute phase of infection (6, 13, 42). Humans appear to be the only host of CCHFV in which disease is manifested. Human infection is usually characterized by a febrile illness with headache, myalgia, and petechial rash, frequently followed by a hemorrhagic state with necrotic hepatitis. The acute stage of the disease in survivors usually lasts from 15 to 20 days, after which the convalescence period begins, characterized by prolonged weakness, weak pulse, confusion, and sometimes temporary complete loss of hair (12, 18, 42).

Up until now, the pathogenesis of CCHF has not been well described, but given the close resemblance between CCHF and Ebola hemorrhagic fever (EHF), it has been hypothesized that CCHF, like EHF, could be a result of the direct injury of virus-infected tissues in combination with the indirect effects of host immune responses (4, 41). Viral load and inflammatory cytokines have recently been implicated in the pathogenesis of the disease (8, 11, 17, 28, 29). However, both factors have so far been studied separately. To further elucidate the role of viral load and cytokines, we present the first study describing the interacting influences of host immune mechanisms and viral load on the severity and outcome of CCHF. The study is based on the differential influences of CCHFV RNA load, antibody response, and pro- and anti-inflammatory cytokine production in sera of patients with confirmed acute CCHF from Kosovo.

(Materials and methods)

Patients and sample collection. Acute-phase serum samples from 46 patients from Kosovo who were diagnosed with CCHFV infection between 2001 and 2007 were included in this study. The patients were treated at the Infectious Disease Clinic, University Clinical Center of Kosovo, Pristina, Kosovo, and clinical data for the patients were collected retrospectively. Patients' blood samples were obtained at the time of admission. Sera were separated from blood cells and...
TABLE 1. Clinical characteristics and laboratory findings for 46 patients with acute CCHF according to disease severity/outcome

| Characteristic/finding | Result for disease severity/outcome of: |
|------------------------|----------------------------------------|
|                        | Moderate (n = 20) | Severe (n = 15) | Fatal (n = 11) |
| No. (%) of patients with bleeding | 16 (80) | 15 (100) | 11 (100) |
| No. (%) of patients requiring transfusion | 0 (0) | 12 (80) | 9 (81.8) |
| Mean lowest platelet count/liter (×10^{12}) (range) | 179.1 (110–280) | 92.5 (20–190) | 80.8 (25–185) |
| No. (%) of patients with hypotension (<100/60 mm Hg) | 7 (35) | 10 (66.7) | 8 (72.7) |
| Highest serum creatinine level (μmol/liter) (range) | 109.8 (52–599) | 108.9 (67–247) | 361.7 (97–513) |
| Highest AST level (U/liter) (range) | 121.4 (24–267) | 508.6 (58–1,616) | 2,653.3 (163–1,255) |
| Highest ALT level (U/liter) (range) | 80.2 (17–218) | 340.5 (41–1,211) | 963 (4–3,552) |
| Longest aPTT (s) (range) | 56.8 (19–123) | 66.8 (22–132) | 131.8 (85–224) |

a AST, aspartate transaminase; ALT, alanine transaminase; aPTT, activated partial thromboplastin time.

RESULTS

Classification of patients according to disease severity. Forty-six patients with confirmed acute CCHFV infection were enrolled in the study. On the basis of their case records, 15 surviving patients were categorized as having severe disease and 20 surviving patients as having moderate disease. In 11 patients, the disease resulted in a fatal outcome. The main clinical characteristics and laboratory values for the patient groups are summarized in Table 1.

Antibody response and CCHFV RNA load. Levels of anti-CCHFV antibodies were measured in all the samples included in the study. Our data show no relationship between the presence of IgM antibodies and clinical classification (P = 0.61), while IgG antibodies were detected in only 5 samples of the patients who survived the disease (Table 2). Although the observed levels of IgM antibodies in patients with fatal outcome were lower than in patients surviving the infection—only 2 (18.2%) patients who died with antibody titers above 1:1,600 came to 12 (34.3%) surviving patients—the difference was not significant (P = 0.3).

Serum samples from 42 patients were tested for the presence of CCHFV RNA, and viral RNA was detected in 40 (95.2%) of them. Viral load ranged from 10^{-2} to 10^{10} copies per ml of serum, depending on the day of illness, the severity of the disease, and the results of the serological analyses (Table 2). Viral RNA could not be detected in 2 patients with moderate disease course. In contrast with the antibody response, viral load seemed to be strongly related to the severity and outcome of the disease. The patients with fatal outcome of CCHF had, on average, higher log viral RNA values (9.31 log_{10} copies/ml; range, 7.76 to 10.05 log_{10} copies/ml) than patients surviving the infection (6.31 log_{10} copies/ml; range, 2.18 to 8.95 log_{10} copies/ml) (P < 0.001). In 9 of the 10 patients with fatal outcomes, detected viral loads were > 1 × 10^{5} copies/ml, while in 30 of the 32 surviving patients, viral RNA loads were < 1 × 10^{4} copies/ml (P < 0.001). Additionally, significantly higher log viral RNA values were detected in patients with severe disease course (mean, 6.97 log_{10} copies/ml; range, 4.39 to 8.95 log_{10} copies/ml) than in patients with moderate disease course (mean, 5.65 log_{10} copies/ml; range, 2.18 to 7.76 log_{10} copies/ml) (P = 0.006) (Fig. 1). Apart from the mean viral load, the change of viral load with time also differed among and surviving nonsurviving patients (P = 0.02) (Fig. 2). While a positive linear dependence of CCHFV RNA load on the day of measurement was observed in the patients with fatal outcome (P = 0.038), a negative linear dependence was present in the surviving patients (P = 0.036), with correlation coefficients of 0.657 and −0.39, respectively. We have focused only on viral load measurements taken up to the 10th day of...
illness, since no longer time frame could be observed in fatal cases. No difference in the changes of viral load with time was observed between patients with severe and moderate disease courses ($P = 0.45$).

When the relationship between detectable antibody response and CCHFV RNA load was analyzed, a significant inverse correlation of viral load with detectable antibody response was observed, with average log viral RNA values highest in patients with no detectable antibodies (7.44 log$_{10}$ copies/ml) and lowest in patients with detectable anti-CCHFV IgM and IgG antibodies (4.01 log$_{10}$ copies/ml) ($P = 0.033$) (Fig. 3A). However, when clinical classification was also taken into

### Table 2. Cytokine levels, CCHFV RNA loads, and anti-CCHFV IgM and IgG antibody titers, by disease course/outcome, in serum samples from patients with acute CCHFV infection

| Disease course/outcome and patient no. | Day of illness | Cytokine level* (pg/ml) | RNA load* (copies/ml) | Antibody titer* |
|----------------------------------------|---------------|-------------------------|----------------------|----------------|
| Moderate                               |               | IL-10 | IL-12 | IFN-γ | TNF-α | IgM | IgG |
| 1                                      | 10            | 8.14  | 38.4  | 7.16  | 12.4  | neg | 800 | neg |
| 2                                      | 7             | 2.99  | 26.9  | 1.88  | 0     | neg | 3,200 | neg |
| 3                                      | 12            | 31.6  | 25.8  | 2.05  | 2.68  | 1.51E+02 | 6,400 | 1,600 |
| 4                                      | 9             | 29.6  | 23.6  | 8.88  | NT$^d$ | 4.20E+02 | 6,400 | 1,600 |
| 5                                      | 12            | 13.2  | 17.1  | 7.17  | 1.14  | 1.15E+05 | >6,400 | neg |
| 6                                      | 3             | 4.54  | 58.8  | 7.86  | 0     | 1.66E+05 | neg | neg |
| 7                                      | 5             | 39.6  | 34.2  | 0     | NT    | 1.71E+05 | neg | neg |
| 8                                      | 5             | 38.6  | 57.2  | 32.8  | 1.94  | 1.90E+05 | 400 | neg |
| 9                                      | 12            | 1.18  | 12.8  | 5.33  | 4.5   | 1.92E+05 | >6,400 | 800 |
| 10                                     | ND$^c$        | 7.84  | 44.5  | 5.8   | 1.84  | 4.25E+05 | neg | neg |
| 11                                     | 6             | 16.1  | 58.2  | 10.5  | 37.7  | 1.19E+06 | neg | neg |
| 12                                     | 4             | 74.1  | 21.8  | 15.7  | 2.68  | 2.47E+06 | 1,600 | neg |
| 13                                     | 4             | 10.5  | 36.4  | 9.17  | 1.69  | 3.07E+06 | 800 | neg |
| 14                                     | 4             | 17.1  | 69.2  | 17.9  | 1.91  | 3.64E+06 | neg | neg |
| 15                                     | 7             | 98.2  | 22    | 6.52  | 3.49  | 2.02E+07 | neg | neg |
| 16                                     | 12            | 68.7  | 98.1  | 23.8  | 2.64  | 5.36E+07 | neg | neg |
| 17                                     | ND$^c$        | 40.9  | 47.6  | 5.97  | NT    | 5.71E+07 | neg | neg |
| 18                                     | 6             | 60.4  | 24.2  | 40.1  | NT    | NT | 1,600 | neg |
| 19                                     | 6             | 134   | 20    | 6.83  | NT    | NT | >6,400 | 100 |
| 20                                     | ND$^c$        | 45.8  | 30.2  | 11.7  | NT    | NT | 3,200 | neg |
| Severe                                 |               |       |       |       |       |    |     |
| 21                                     | 5             | 65.2  | 21.2  | 0.37  | 9.43  | 2.47E+04 | 1,600 | neg |
| 22                                     | 6             | 55.8  | 26    | 7.82  | 8.33  | 9.35E+05 | >6,400 | >6,400 |
| 23                                     | ND$^c$        | 18.4  | 10.9  | 2.9   | 0     | 1.27E+06 | >1,600 | neg |
| 24                                     | 8             | 194   | 21.8  | 18.3  | 1.14  | 2.67E+06 | >6,400 | neg |
| 25                                     | 6             | 162   | 19.3  | 11.2  | 8.44  | 2.79E+06 | 3,200 | neg |
| 26                                     | 6             | 51.8  | 24.2  | 2.98  | 3.16  | 4.63E+06 | neg | neg |
| 27                                     | 15            | 15.3  | 27.7  | 26.7  | 2.24  | 6.54E+06 | neg | neg |
| 28                                     | 5             | 10.3  | 20.1  | 44.8  | 0.95  | 1.34E+07 | neg | neg |
| 29                                     | ND$^c$        | 122   | 155   | 91    | NT    | 1.36E+07 | neg | neg |
| 30                                     | ND$^c$        | 119   | 31.5  | 41    | 6.55  | 2.10E+07 | neg | neg |
| 31                                     | 4             | 51    | 18.1  | 4.1   | NT    | 2.19E+07 | neg | neg |
| 32                                     | 5             | 115   | 33.8  | 18.6  | 7.41  | 2.70E+07 | neg | neg |
| 33                                     | 11            | 210   | 19.3  | 61.1  | 5.39  | 3.33E+07 | >6,400 | neg |
| 34                                     | 4             | 44.6  | 6.72  | 5.63  | 6.28  | 7.04E+08 | neg | neg |
| 35                                     | 2             | 18.9  | 15.6  | 26.2  | NT    | 8.86E+08 | neg | neg |
| Fatal                                  |               |       |       |       |       |    |     |
| 36                                     | 8             | 151   | 35.8  | 7.04  | NT    | NT | 6,400 | neg |
| 37                                     | ND$^c$        | 486   | 48    | 29.2  | 25.2  | 5.72E+07 | 1,600 | neg |
| 38                                     | 3             | 42.2  | 30.2  | 27.4  | 20.4  | 4.92E+08 | neg | neg |
| 39                                     | ND$^c$        | 220   | 2.52  | 20.6  | 18.2  | 1.00E+09 | 800 | neg |
| 40                                     | 3             | 44.1  | 18.5  | 5.97  | 39.1  | 1.32E+09 | neg | neg |
| 41                                     | 7             | 147   | 11.5  | 33.3  | 6.75  | 2.72E+09 | 400 | neg |
| 42                                     | 4             | 94    | 0     | 8.11  | 8.4   | 2.79E+09 | neg | neg |
| 43                                     | 6             | 266   | 63.2  | 89.6  | 43.2  | 6.76E+09 | 3,200 | neg |
| 44                                     | 9             | 377   | 62.1  | 219   | 13.6  | 7.51E+09 | neg | neg |
| 45                                     | 7             | 292   | 88.6  | 186   | NT    | 7.63E+09 | neg | neg |
| 46                                     | 6             | 259   | 19.4  | 25.9  | NT    | 1.13E+10 | 400 | neg |

* Determined by ELISA.

$^b$ Determined by quantitative real-time RT-PCR.

$^c$ ND, no data.

$^d$ NT, not tested.

$^e$ neg, negative.
account, it was shown that an inverse correlation could be observed only in patients surviving the infection \((P = 0.003)\), while no change in viral RNA values with the development of antibody response was detected in patients with fatal outcome of CCHF \((P = 0.76)\) (Fig. 3B).

Cytokine levels. Concentrations of IL-10, IL-12, and IFN-\(\gamma\) were determined in all serum samples. However, due to the limited amount of available samples, levels of TNF-\(\alpha\) could be measured in only 34 serum samples (Table 2). Elevated levels of IL-10, IFN-\(\gamma\), and TNF-\(\alpha\) were detected in almost all the samples tested, regardless of the clinical course of the disease. In contrast, concentrations of IL-12 were significantly lower than IL-12 concentrations measured in healthy controls \((P < 0.001)\) (data not shown). Considerable differences in cytokine concentrations were observed among patient groups (Fig. 4). In patients with fatal outcome of CCHF, significantly higher serum levels of IL-10 (mean, 216.2 pg/ml; range, 42.2 to 486.0 pg/ml) \((P < 0.001)\), IFN-\(\gamma\) (mean, 59.3 pg/ml; range, 6.0 to 219.0 pg/ml) \((P = 0.015)\), and TNF-\(\alpha\) (mean, 22.6 pg/ml; range, 6.8 to 43.2 pg/ml) \((P < 0.001)\) were detected, compared with cytokine levels in patients who survived the infection. No differences in serum IL-12 levels between patients who died (mean, 34.5 pg/ml; range, 0 to 88.6 pg/ml) and those who survived the infection \((P = 1)\) were detected. In addition, differences in cytokine concentrations between the groups of patients who survived the infection were observed. Significantly higher levels of IL-10 were detected in patients with severe clinical course of disease (mean, 83.6 pg/ml; range, 10.3 to 210.0 pg/ml) than in patients with moderate disease course (mean, 37.1 pg/ml; range, 1.2 to 134.0 pg/ml) \((P = 0.016)\). By contrast, serum IL-12 concentrations were significantly higher in patients with moderate disease course (mean, 38.4 pg/ml; range, 12.8 to 98.1 pg/ml) than in patients with severe disease course (mean, 28.6 pg/ml; range, 0 to 155.0 pg/ml) \((P = 0.013)\). No significant differences were observed in measured levels of IFN-\(\gamma\) and TNF-\(\alpha\) among patients with severe disease course (means, 24.2 pg/ml [range, 0.4 to 91.0 pg/ml] and 4.9 pg/ml [range, 0 to 9.4 pg/ml], respectively) and patients with moderate disease course (means, 11.4 pg/ml [range, 0 to 40.1 pg/ml] and 5.3 pg/ml [range, 0 to 37.7 pg/ml], respectively) \((P = 0.214\) and 0.252, respectively).

The relationship between viral RNA load and cytokine concentrations was also analyzed. When cytokine levels were compared with viral load concentrations, significant positive correlations were observed for IL-10, IFN-\(\gamma\), and TNF-\(\alpha\), with correlation coefficients of 0.587, 0.631, and 0.448, respectively \((P < 0.001, < 0.001,\) and \(0.004,\) respectively). No such correlation was observed for IL-12 (Fig. 5).

DISCUSSION

The pathogenesis of CCHF is poorly understood. As in other VHF, CCHFV targets and impairs cells that initiate the antiviral immune response, most likely leading to overwhelming viral burdens and immune and vascular dysregulation (13, 20). In this study, viral load seemed to be strongly related to clinical classification, with higher viral loads detected in patients with fatal outcomes than in surviving patients. Also, in patients with fatal outcome, a detectable antibody response was weaker, if at all present. These results are in accordance with our previous findings (11), as well as with other CCHF studies and studies on other VHF, like Ebola, Lassa, and yellow fevers, where high viral loads have been detected in patients with fatal outcomes than in surviving patients. Also, in patients with fatal outcome, a detectable antibody response was weaker, if at all present. These results are in accordance with our previous findings (11), as well as with other CCHF studies and studies on other VHF, like Ebola, Lassa, and yellow fevers, where high viral loads have been detected in patients with fatal outcomes, often with no detectable antibody response (8, 20, 29, 31, 33, 37). Results of this study also confirm our previous findings on a smaller number of patients (11) that a viral load of \(>10^9\) copies/ml is a strong factor for differentiating CCHF patients who died from those who survived. Based on these studies, we believe that viral load could be used as a criterion in deciding on the initiation of antiviral
therapy with ribavirin, since the drug is most efficient only when administered early in the course of the disease and because the decision of which patients should be treated may be difficult (14, 16, 24). In addition to our previous findings, we have shown that CCHFV viral load values correlate not only with disease outcome but also with disease severity in surviving patients. In particular, significantly lower viral loads were detected in patients with moderate disease course than in patients with severe disease course. Such correlation between viremia levels and disease severity has also been reported in patients with dengue virus infection and in patients with hemorrhagic fever with renal syndrome caused by Dobrava virus (23, 32, 39, 40). Furthermore, a significant difference between changes of viral load with time for nonsurviving and surviving patients was observed. In patients with fatal outcome, the viral load actually increased with time, while a decrease was observed in surviving patients. However, it is important to note that only one sample was taken from each patient and thus the viral load concentrations observed at different time points derive from different patients. Similar findings have been described by Towner et al. in their study of Ebola patients (37). Also, while the development of the detectable antibody response was significantly associated with a decrease in CCHFV RNA load in surviving patients, no such association was observed in patients with fatal outcome. A delayed immune response is most likely an important factor in the pathogenesis of CCHF, leading to uncontrolled replication and spread of the virus throughout the body.

One of the main characteristics of VHF is an impairment of endothelial cell function leading to changes in vascular permeability and hemorrhage. However, the endothelial damage is most likely not a result of the direct effects of virus replication but rather a result of multiple host-induced mechanisms, including cytokines (20, 25, 35). Indeed, recent studies on cytokine profiles in CCHF patients suggest that proinflammatory cytokines play a role in the pathogenesis of the disease (17, 28).

In these studies, serum levels of proinflammatory cytokines IL-6 and TNF-α were found to be higher in patients with fatal CCHF than in patients with nonfatal CCHF, whereas there was no significant difference in levels of regulatory cytokine IL-10 between the two groups. This is in contrast with our findings. However, the numbers of patients included in the mentioned studies are lower than the number in our study and patient groups are not proportional. Thus, on the basis of the results of our study, we hypothesize that severe disease course and even fatal outcome in CCHF patients could be associated with immunosuppression, which blocks the appropriate cell-mediated immune response early in the course of the disease. Significantly higher levels of IL-10 were detected in patients with fatal outcome than in surviving patients. Also, for surviving patients, concentrations of IL-10 were higher in patients with severe disease course than in patients with moderate disease course. Such correlation between increased serum levels of IL-10 and poor outcome has also been observed in patients with Ebola and dengue hemorrhagic fevers (2, 21, 38). In addition, increased release of IL-10, IL-6, and TNF-α from CCHFV-infected monocyte-derived dendritic cells compared to that from uninfected cells was recently demonstrated in an in vitro study (10). Interesting observations of our study were significantly reduced levels of IL-12 in all CCHF patients. IL-12 is one of the main inductors of cell-mediated immunity and is downregulated by IL-10 (1). This further supports our hypothesis on IL-10-mediated immunosuppression, which in turn could lead to an increase in CCHFV viremia, demonstrated also by our findings on viral RNA load. In addition, a positive linear dependence between viral load and IL-10 levels was observed. In our opinion, a rise in viremia might consequently cause an even stronger inflammatory response. In particular, we observed higher levels of proinflammatory cytokines IFN-γ and TNF-α in patients with fatal outcomes of CCHF. A positive linear dependence between viral load and these cytokines was observed as well. Contribution of a
strong inflammatory response to the pathogenesis of CCHF has also been suggested in recent studies demonstrating hemophagocytosis and high neopterin serum levels in CCHF patients (7, 27).

Based on the obtained results, we suggest that a combination of high levels of regulatory cytokine IL-10, which inhibits cell-mediated immunity by downregulating IL-12 expression, with high levels of proinflammatory cytokines IFN-γ and TNF-α might play an important role in the pathogenesis of CCHF. Very similar findings, with overproduction of both proinflammatory cytokines (IFN-γ and TNF-α) and anti-inflammatory cytokines (IL-10 and IL-6) and relatively low levels of IL-12 have been observed in patients with dengue virus infection, another viral illness characterized by plasma leakage and an increase in microvascular permeability (19, 21, 23, 26, 30). The importance of a strong regulatory cytokine response leading to increased viremia and consequently to a strong, possibly systemic inflammatory response has also been implicated in the pathogenesis of other VHF, such as Lassa and Ebola (3, 34, 38).

Cytokines, chemokines, and other inflammatory mediators function in a pleiotropic manner, acting on many different cell types to regulate the host’s immune response. Typically, their antimicrobial actions are confined to areas of infection. However, when present in high concentrations, they might act systemically and have toxic or even lethal effects. A dysregulation of cytokine and chemokine responses is thought to cause capillary leakage syndrome, which is a prominent characteristic of VHF (1, 20).

To conclude, the results of our study favor the hypothesis that CCHF could be a result of a delayed and downregulated immune response caused by IL-10, which leads to increased replication and spread of CCHFV throughout the body. This consequently triggers an increased production of IFN-γ and TNF-α, cytokines mediating vascular dysfunction, disseminated intravascular coagulation, organ failure, and shock when

![Disease course/outcome](http://cvl.asm.org/downloaded-from.png)

**FIG. 4.** Comparison of serum IL-10, IL-12, IFN-γ, and TNF-α concentrations and clinical course/outcome of the disease in patients with acute CCHF. Horizontal bars show median values. Dotted lines represent the upper limits of normal values.
present in high concentrations. Further studies, preferably including serial patient samples, are needed to test our hypothesis. Also, more factors are undoubtedly involved in the pathogenesis of CCHF and are waiting to be determined.

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