Temporal Relationship of Viral Load, Ribavirin, Interleukin (IL)–6, IL-8, and Clinical Progression in Patients with Severe Acute Respiratory Syndrome

Wei-Kung Wang,1,4 Shey-Ying Chen,1,6 I.-Jung Liu,1,6 Chuan-Liang Kao,7 Hui-Ling Chen,1 Bor-Liang Chiang,1 Jann-Tay Wang,1 Wang-Hwei Sheng,1 Po-Ren Hsueh,7 Chao-Fu Yang,1 Pan-Chyr Yang,1 Shan-Chwen Chang,1 and the Severe Acute Respiratory Syndrome Research Group of the National Taiwan University (NTU) College of Medicine/NTU Hospital

1Institute of Microbiology, 2Medical Technology, College of Medicine, National Taiwan University, and Departments of 3Emergency Medicine, 4Internal Medicine, and 5Pediatrics, National Taiwan University Hospital, Taipei, Taiwan

Although viral replication and overwhelming immune responses are believed to contribute to the progression of severe acute respiratory syndrome (SARS), little is known about the temporal relationship between viral load, ribavirin, proinflammatory cytokines, and clinical progression. We report that ribavirin was not effective in reducing the SARS coronavirus load in 3 of 8 probable cases studied and that elevated levels of interleukin (IL)–6 and IL-8 subsequent to the peak viral load were found in 8 and 6 cases, respectively. The nadir lymphocyte count during lymphopenia, the peak level of lactate dehydrogenase, and the peak density of pulmonary infiltrates lag further behind the peak viral load by a median of 4, 5, and 3.5 days, respectively. These findings provide important information for therapeutic strategies to treat SARS.

Severe acute respiratory syndrome (SARS) is an emerging infectious disease that poses a major threat to the health of people worldwide [1, 2]. The etiological agent is a novel coronavirus: SARS-associated coronavirus (SARS-CoV) [2–4]. After infection with the SARS-CoV, there is an incubation period ranging from 2 to 10 days, followed by a wide spectrum of symptoms and signs with the characteristic presentations of fever, dyspnea, progressively changing radiographic findings, and/or respiratory failure [2, 5, 6]. A recent study reported that decreases in SARS-CoV load preceded disease progression and suggested that overexuberant immune responses, rather than uncontrolled viral replication, may contribute to the progressive damage to the lung [7]. Pathological investigation of SARS pneumonia has revealed hemophagocytosis, which is reminiscent of pneumonia due to H5N1 influenza virus, and suggests that proinflammatory cytokines are dysregulated [8]. However, little is known about the temporal relationship between viral load, cytokine dysregulation, and clinical progression. Moreover, the effect of ribavirin therapy on viral load in vivo remains largely unclear. Using quantitative real-time RT-PCR, we measured the SARS-CoV load in sequential throat-wash specimens obtained from patients with probable SARS and examined its relationship with ribavirin, proinflammatory cytokines, and disease progression. Throat-wash specimens were chosen because they are reported to have a high positivity rate for SARS-CoV among patients with probable SARS [3, 9]. From a technical perspective, throat-wash specimens are easier to collect than are currently recommended specimens, including nasopharyngeal aspirates and swab specimens and oropharyngeal swab specimens.

Methods. The study included 8 adult patients, all of whom met the World Health Organization clinical case definitions of probable SARS [10] and were admitted to the negative-pressure ventilated room at the National Taiwan University Hospital (Taipei) between 16 April and 26 April 2003 during the SARS outbreak in Taipei. All cases of SARS were confirmed by laboratory testing [10]. The first day of fever is defined as day 1 of illness. Sequential chest radiography and routine laboratory tests were performed at least twice per week. Oral ribavirin was given for 10 days unless adverse effects were noted, and methylprednisolone was administered during the second week for most patients or during the first week for patients with a rapidly progressing disease course, as described elsewhere [11]. Intravenous immunoglobulin (IVIG) was given to patients with severe leukopenia, thrombocytopenia, or marked progression of lung lesions [11].

With the consent from each patient, throat-wash specimens, obtained by gargling 10 mL of normal saline, were collected every other day during the first 2 weeks of hospitalization and every 5 days thereafter or until discharge, according to the guidelines for aerosol-generating procedures [12]. All samples were transferred to the biosafety level 3 laboratory and stored at −80°C until use. After thawing, 5-mL throat-wash specimens were centrifuged at 450 g for 15 min to obtain the supernatant,
from which 560 μL was subjected to viral RNA extraction [9]. An aliquot of viral RNA and a known amount of the in vitro-transcribed RNA were quantified by a real-time RT-PCR assay described elsewhere [9]. The lower limit of detection was 90 copies per mL of throat wash. Levels of proinflammatory cytokines—IL-6, IL-8, and TNF-α—were measured by commercial ELISA kits (Endogen).

**Results.** The demographic information and laboratory findings for the 8 patients with SARS are summarized in table 1. Among the laboratory tests that have been reported to have abnormal results in cases of SARS, lymphopenia and elevated lactate dehydrogenase (LDH) levels were found in all cases examined [2, 5, 6]. The nadir lymphocyte count in patients with lymphopenia occurred at a median of day 10 (range, day 8–13) of illness, and the peak LDH level occurred at a median of day 12 (range, day 9–18) of illness. Sequential chest radiographs for each patient were also evaluated by radiologists to determine the radiograph with maximum pulmonary infiltrates, which occurred at a median of day 10.5 (range, day 6–13) of illness.

Viral loads in sequential throat-wash samples obtained from each patient were next examined (table 1). The peak viral loads ranged from $3.34 \times 10^4$ to $7.54 \times 10^4$ copies/mL, which was within the range reported elsewhere [7]. There was no correlation between the peak viral load and the nadir lymphocyte count or peak LDH level ($r = 0.694$ and 0.175 and $P = .084$ and .679, respectively, by simple linear regression). The peak viral load occurred at a mean of day 6.9 (range, day 4–11) of illness, which was earlier than day 10, which was reported elsewhere [7]. Of interest, the timing of the 3 parameters of clinical progression—nadir lymphocyte count, peak LDH level, and maximum pulmonary infiltrates—lagged behind the peak viral load by a median of 4 days, 5 days, and 3.5 days, respectively. These findings indicate that SARS progresses after the viral loads decrease.

To evaluate the effect of ribavirin therapy in vivo, we examined the viral load pattern and its relationship to ribavirin use. As shown in figure 1, although, as described elsewhere, the “inverted V-shape” viral load pattern with primarily a single peak was seen in 6 patients (patients A–F), a pattern with 2 peaks was found in the other 2 (patients G and H) [7]. This finding suggests that the viral load patterns vary among different patients and that some patients have a more protracted and complex profile. When comparing these findings with ribavirin use, a decrease in the viral load was found in 4 cases (patients A, B, C, and F). However, viral load remained high despite >10 days of ribavirin therapy, in patients D, E, and H. In patient G, the viral load decreased before the administration of ribavirin, and then increased and decreased after ribavirin was received. This finding suggests that ribavirin was not effective in reducing the viral load in 3 of the 8 patients studied.

We next investigated the temporal relationship of peak viral load, proinflammatory cytokine levels (including IL-6, IL-8, and TNF-α), and progression of lung lesion. In contrast to the IL-6 and IL-8 levels, the TNF-α level was not detectable in most patients and, therefore, was not analyzed. The elevation in the IL-8 level generally paralleled that of the IL-6 level, although a delayed increase (between days 18 and 20 of illness) was found in 3 cases (patients D, F, and H) in the absence of nosocomial infection or other identified insults (figure 1). Overall, the peak IL-6 and/or elevated IL-8 levels coincided with or after the peak viral load and preceded or concurred with the maximum pulmonary infiltrates (figure 1). Of note, the elevation of the IL-6 levels associated with the second viral load peak.

**Table 1.** Demographic, laboratory, and radiographic features of study subjects with severe acute respiratory syndrome (SARS).

| Patient | Age in years | Sex | Cells/µL | Time of occurrence, day of illness | Peak lactate dehydrogenase level | Maximum pulmonary infiltrates | Peak SARS-CoV load |
|---------|--------------|-----|----------|----------------------------------|---------------------------------|-------------------------------|-------------------|
| A       | 48           | F   | 613      | 12                               | 529                             | RUL patch                     | $1.99 \times 10^4$ |
| B       | 26           | M   | 567      | 8                                | 1498                            | Bil patch                      | $7.81 \times 10^4$ |
| C       | 25           | F   | 729      | 13                               | 782                             | RLL/LLL patch                  | $9.97 \times 10^3$ |
| D       | 52           | M   | 126      | 10                               | 916                             | RML/RLL patch                  | $7.54 \times 10^5$ |
| E       | 48           | M   | NA       | NA                               | 480                             | RUL/LLL patch                  | $1.19 \times 10^4$ |
| F       | 47           | M   | 383      | 13                               | 788                             | RLL/LLL patch                  | $1.86 \times 10^4$ |
| G       | 26           | F   | 450      | 10                               | 773                             | RLL patch                      | $3.34 \times 10^{3a}$ |
| H       | 28           | F   | 310      | 10                               | 931                             | Bil patch                      | $4.69 \times 10^{3a}$ |

**NOTE.** The diagnosis of SARS was based on the World Health Organization clinical definitions [10]. Bil, bilateral; LLL, left lower lung; NA, not available; RLL, right lower lung; RML, right middle lung; RUL, right upper lung.

*Two peak loads were observed. The first is shown, to indicate the extent of initial viral replication.*
Figure 1. Time course of viral load, ribavirin use, proinflammatory cytokine (IL-2 and IL-8) level, and pulmonary progression in patients with severe acute respiratory syndrome (SARS). A–H. Data for 8 patients with probable SARS. See Methods for a description of the assays used in these analyses. Closed triangle, intubation; dashed lines, lower limit of viral load detection (90 RNA copies/mL); hatched bars, ribavirin use; open bars, steroid use; open triangles and quadrangle, times at which maximum pulmonary infiltrates were detected by chest radiography; stippled bars, intravenous immunoglobulin use.
was not observed in patients G and H, probably because of steroid use on day 9 and day 11, respectively. The temporal relationship between peak viral load and IL-6, as well as the subsequent development of maximum pulmonary infiltrates, the nadir lymphocyte count, and the peak LDH level, is summarized in figure 2. These findings suggest that initial viral replication leads to activation of proinflammatory cytokines that, together with other factors, contribute to disease progression.

Discussion. It has been reported that ribavirin cannot inhibit the replication of SARS-CoV in vitro [13]. Previously, Peiris et al. [7] examined the viral load pattern in 14 patients who received ribavirin and steroid therapy. Because the viral load was not analyzed with respect to the exact time course of therapy for each patient, the effect of ribavirin on viral load was not addressed. In this study, we examined the effect of ribavirin on viral load in vivo. Despite the small sample size studied, we closely monitored the viral load and ribavirin use in each patient. We report different profiles of viral load during the course of infection and that use of ribavirin does not result in reduction of viral load in ~40% of the patients studied. Whether ribavirin use is associated with the high mortality among patients with SARS requires further investigation.

This is the first study investigating the temporal relationship of viral load, proinflammatory cytokines, and clinical progression. Although there was some variation in the cytokine profiles, our analysis revealed a common picture and indicated that the elevation of IL-6 and/or IL-8 levels subsequent to the peak of viral replication is involved in pulmonary progression (figure 1). Similarly, the occurrence of the nadir lymphocyte count and peak LDH level lagged behind the peak viral load and IL-6 level (figure 2). This suggests that an immune-mediated process is involved in lymphopenia and tissue damage in patients with SARS [14]. Of note, the nadir lymphocyte count seems to correlate with the peak viral load ($P = .084$), raising the possibility of a direct effect of viral replication on lymphopenia. Taken together, our findings support the hypothesis of a viral replication phase followed by an immune response phase in the pathogenesis of SARS. Moreover, these findings provide important information on therapeutic strategies for SARS, including antivirals and immune-based regimens, such as steroids, IVIG, and convalescent-phase serum infusions. With regard to convalescent-phase serum infusions, we also examined IgG seroconversion in these 8 patients by an indirect immunofluorescence assay described elsewhere [15]. As shown in figure 1, seroconversion correlated with the decrease in viral load in most cases. However, a delay in the decrease of viral load was found in patients E and F, and the viral load remained high in patients D and H in the presence of antibody. Although a study preliminarily suggested the efficacy of convalescent-phase serum infusions [16], our observation suggests that development of antibody may not correlate with clearance of virus, which should be taken into consideration before using convalescent-phase serum infusions as a therapeutic strategy.

Acknowledgments

We thank all medical personnel at the National Taiwan University Hospital, for caring for patients with severe acute respiratory syndrome (SARS) during the outbreak in Taipei, and all other members of the SARS research group of the National Taiwan University (NTU) College of Medicine/NTU Hospital, including Ding-Shinn Chen, Yuan-Teh Lee, Hong-Neng Ho, Chu-Min Teng, Jin-Town Wang, Pei-Jer Chen, Li-Min Huang, and Ming-Fu Chang, for discussion and coordination; Tun-Hou Lee at the Harvard School of Public Health (Boston), for critical comments; and Applied Biosystems (Taiwan), for providing the ABI prism 7000 sequence detector during the outbreak.

Financial support. National Science Council Taiwan (grant NSC92-3112-B-002-032).

Conflict of interest. All authors: No conflict.

References

1. World Health Organization. Summary of probable SARS cases with onset of illness from 1 November 2002 to 31 July 2003. 26 September 2003. Available at: http://www.who.int/csr/sars/country/2003_09_26/en/. Accessed 26 December 2003.
2. Peiris JS, Yuen KY, Osterhaus AD, Stohr K. The severe acute respiratory syndrome. N Engl J Med 2003; 349:2431–41.
3. Ksiazek TG, Erdman D, Goldsmith C, et al. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med 2003; 348:1953–66.
4. Drosten C, Gunther S, Preiser W, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med 2003; 348:1967–76.
5. Lee N, Hui D, Wu A, et al. A major outbreak of severe acute respiratory syndrome in Hong Kong. N Engl J Med 2003; 348:1986–94.
6. Booth CM, Matukas LM, Tomlinson GA, et al. Clinical features and short-term outcomes of 144 patients with SARS in greater Toronto area. JAMA 2003; 289:2801–9.
7. Peiris JSM, Chu CH, Cheng VCC, et al. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. Lancet 2003; 361:1767–72.
8. Nicholls JM, Poon LLM, Lee KC, et al. Lung pathology of fatal severe acute respiratory syndrome. Lancet 2003; 361:1773–8.
9. Wang WK, Chen SY, Liu JJ, et al. Detection of large amount of SARS-associated coronavirus in throat wash and saliva: implications for transmission and early diagnosis. Emerg Infect Dis 2004; 10:1213–9.
10. World Health Organization. Case definitions for surveillance of SARS, Geneva: 1 May 2003. Available at: http://www.who.int/csr/sars/case_definition/en/. Accessed on 26 December 2003.
11. Wang JT, Sheng WH, Fang CT, et al. Clinical manifestations, laboratory findings and treatment outcomes of SARS patients. Emerg Infect Dis 2004; 10:818–24.
12. U.S. Centers for Disease Control and Prevention. Interim domestic infection control precautions for aerosol-generating procedures on patients with severe acute respiratory syndrome (SARS). 20 May 2003. Available at: http://www.cdc.gov/ncidod/sars/aerosol/infectioncontrol.htm. Accessed on 4 October 2003.
13. Cinatl J, Morgenstern B, Bauer G, Chandra P, Rabenau H, Doerr HW. Glycyrrhizin, an active component of liquorice roots, and replication of SARS-associated coronavirus. Lancet 2003; 361: 2045–6.
14. Wong RSM, Wu A, To KF, et al. Haematological manifestations in patients with severe acute respiratory syndrome: retrospective analysis. BMJ 2003; 326:1558–62.
15. Hsueh PR, Hsiao SH, Yeh SH, et al. Microbiologic characterization, serologic responses, and clinical manifestations in severe acute respiratory syndrome, Taiwan. Emerg Infect Dis 2003; 9:1163–7.
16. Soo YO, Cheng Y, Wong R, et al. Retrospective comparison of coalescent plasma with continuing high dose of methylprednisolone treatment in SARS patients. Clin Microbiol Infect 2004; 10:678–8.