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

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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 3.34×10^3 to 7.54×10^5 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 concurred 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 | Nadir lymphocyte count | | Peak lactate dehydrogenase level | | Maximum pulmonary infiltrates | | Peak SARS-CoV load | |
			Cells/μL	Time of occurrence, day of illness	U/L	Time of occurrence, day of illness	Chest radiograph findings, location/type of lesion	Time of occurrence, day of illness	RNA copies per mL of throat wash	Time of occurrence, day of illness
A	48	F	613	12	529	18	RUL patch	12	1.99×10^4	8
B	26	M	567	8	1498	12	Bil patch	10, 12	7.81×10^4	5
C	25	F	729	13	782	12	RLL/LLL patch	13	9.97×10^3	11
D	52	M	126	10	916	12	RML/RLL patch	10	7.54×10^5	6
E	48	M	NA	NA	480	10	RUL/LLL patch	6	1.19×10^4	6
F	47	M	383	13	788	9	RLL/LLL patch	10	1.86×10^4	7
G	26	F	450	10	773	12	RLL patch	10	3.34×10^{3a}	8
H	28	F	310	10	931	10	Bil patch	12	4.69×10^{3a}	4

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.

[a] Two peak loads were observed. The first is shown, to indicate the extent of initial viral replication.
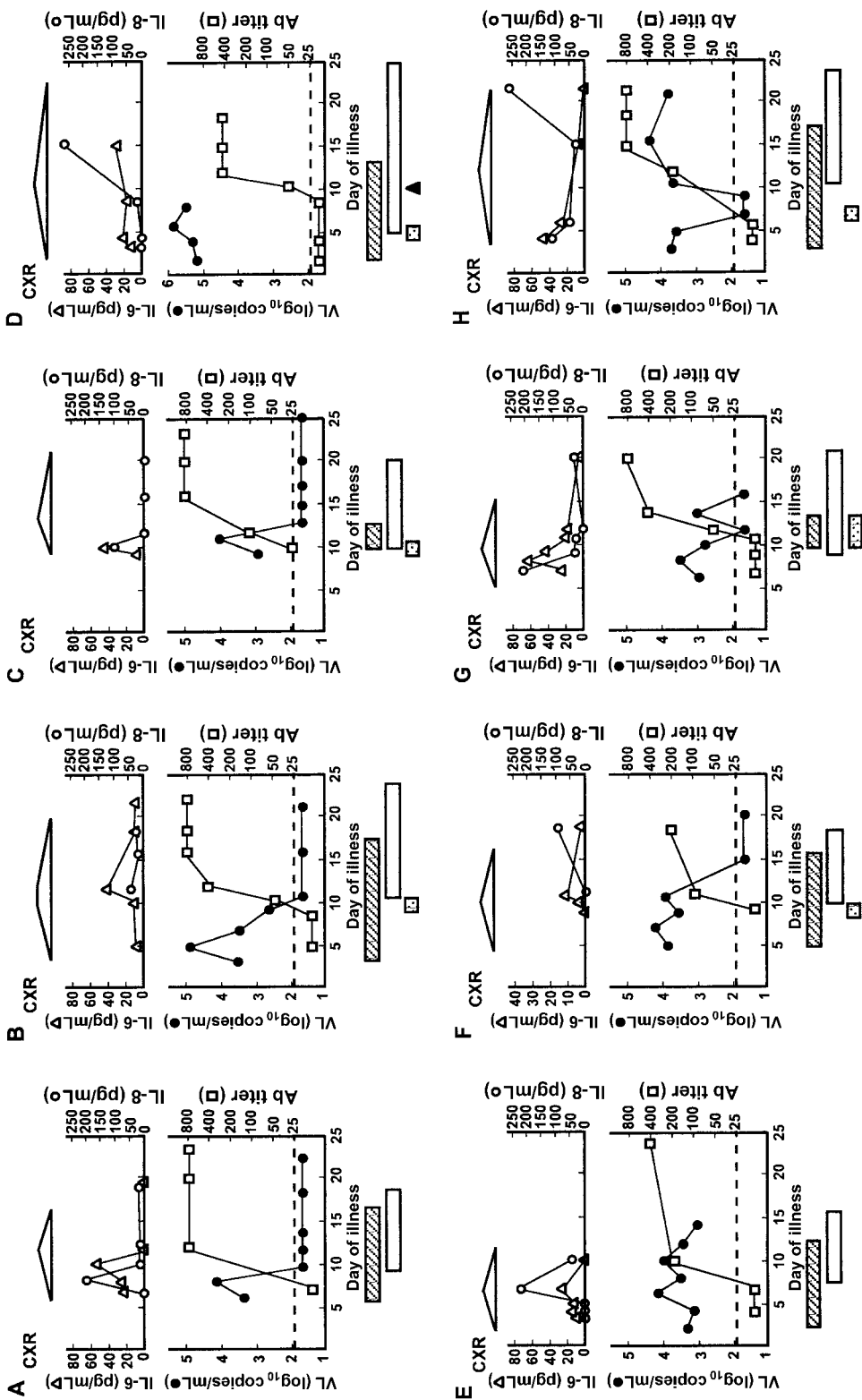
Figure 1. Time course of viral load, ribavirin use, proinflammatory cytokine (IL-2 and IL-8) level, and pulmonary progression in patients with severe acute respiratory syndrome (SARS). *A–H,* Data for 8 patients with probable SARS. See Methods for a description of the assays used in these analyses. *Closed 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.
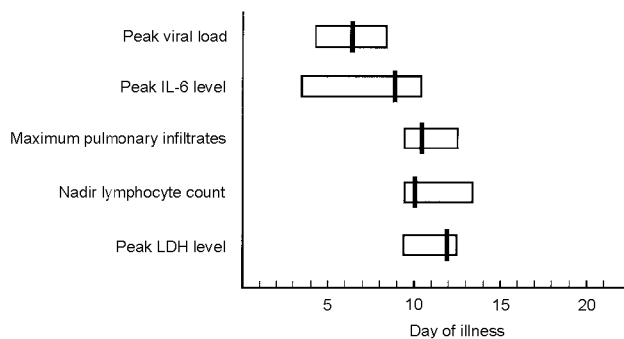
Figure 2. Temporal relationship of peak viral load, peak IL-6 level, maximum pulmonary infiltrates, nadir lymphocyte count, and peak lactate dehydrogenase (LDH) level in patients with severe acute respiratory syndrome. *Boxes,* interquartile range; *vertical lines,* median day of peak or nadir level.

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.

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