

Analysis of Serum Cytokines in Patients with Severe Acute Respiratory Syndrome

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Severe acute respiratory syndrome (SARS) is an acute infectious disease of the respiratory system. Although a novel coronavirus has been identified as the causative agent of SARS, the pathogenic mechanisms of SARS are not understood. In this study, sera were collected from healthy donors, patients with SARS, patients with severe SARS, and patients with SARS in convalescence. The serum concentrations of interleukin-1 (IL-1), IL-4, IL-6, IL-8, IL-10, tumor growth factor beta (TGF- β), tumor necrosis factor alpha (TNF- α), and gamma interferon (IFN- γ) were measured by enzyme-linked immunosorbent assays (ELISA). The concentrations of IL-1 and TNF- α were not significantly different in different groups. The IL-6 concentration was increased in SARS patients and was significantly elevated in severe SARS patients, but the IL-6 concentrations were similar in convalescent patients and control subjects, suggesting that there was a positive relationship between the serum IL-6 concentration and SARS severity. The concentrations of IL-8 and TGF- β were decreased in SARS patients and significantly reduced in severe SARS patients, but they were comparable in convalescent SARS patients and control subjects, suggesting that there was a negative relationship between the IL-8 and TGF- β concentrations and SARS severity. The concentrations of IFN- γ , IL-4, and IL-10 showed significant changes only in convalescent SARS patients. The IFN- γ and IL-4 levels were decreased, while the levels of IL-10 were increased, and the differences between convalescent SARS patients and other patient groups were statistically significant. These results suggest that there are different immunoregulatory events during and after SARS and may contribute to our understanding of the pathogenesis of this syndrome.

Severe acute respiratory syndrome (SARS) is an acute infectious disease of the respiratory system. Although the World Health Organization has announced that a novel coronavirus was identified as the infectious agent that causes SARS (7, 16), there are no specific and efficient clinical treatments for SARS since the pathogenesis of SARS is not understood.

The clinical presentation indicates that acute lung injury is the major clinical characteristic of SARS and leads to acute respiratory distress syndrome (ARDS) in some severe SARS patients. Lung pathological examination has indicated that there are necrosis, shed bronchial and alveolar epithelial cells, hyaline membrane formation, and extravasation of erythrocytes and macrophages in alveolar spaces (20). The acute lung injury is a complex and multifactorial pathophysiological process involving cytokines and adhesion molecules, as well as inflammatory and immune cells. It has been demonstrated that some proinflammatory cytokines, such as tumor necrosis factor alpha (TNF- α) and interleukin-1 (IL-1), play a key role in the pathogenesis of acute lung injury in several pathological conditions, such as sepsis, severe burns, and acute pancreatitis (9, 10). In some patients with viral infections of the respiratory system, such as influenza, the cytokine TNF- α is also an important mediator in the pathogenesis (6, 13). However, the

mechanisms of the novel coronavirus-induced infection ARDS remain unclear so far. Mark Selter, an officer of the World Health Organization, has inferred that a severe immune response kills SARS victims and that some cytokines may play an important role in the process (18). However, this hypothesis has not been supported by any clinical or experimental evidence.

Cytokines are widely recognized as important mediators of the inflammatory response. Some cytokines, including IL-6, IL-8, TNF- α , IL-1, gamma interferon (IFN- γ), and tumor growth factor beta (TGF- β), promote inflammation by inducing cell injury, while other cytokines, such as IL-4 and IL-10, counteract this process. It has been reported that circulating T lymphocyte counts were decreased in patients with SARS at the early stage, especially for the CD4 and CD8 subsets, and the decrease was associated with disease severity, indicating that some immune functional changes occurred in SARS patients (24). Therefore, analysis of cytokines in the sera of patients with SARS is important for understanding the pathogenesis of SARS and the mechanisms of the acute lung injury.

In this study, the levels of eight cytokines, including TNF- α , IL-1 α , IL-4, IL-6, IL-8, IL-10, TGF- β , and IFN- γ , were measured by enzyme-linked immunosorbent assays (ELISA) in sera from control donors, patients with SARS, patients with severe SARS, and convalescent SARS patients. The results showed that the levels of some cytokines were significantly changed in patients with SARS and were associated with disease severity. These data may be helpful for understanding the pathogenesis of SARS.

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TABLE 1. Basic information for serum donors

Group	n	No. of males	No. of females	Ages (yr)		Intensive care unit	Time (days)	Complications	No. of deaths
				Range	Mean				
Control	20	8	12	21–49	34.8				
SARS	30	19	11	17–80	44.1	No	3–6	No	0
Severe SARS	30	21	9	19–86	45.4	Yes	3–7	No	4
Convalescent SARS	30	15	15	14–68	35.3	No	35–42	No	0

MATERIALS AND METHODS

Patient selection. China-Japan Friendship Hospital became the specialized hospital for SARS patients during a SARS outbreak in the Beijing area. A total of 228 patients with SARS were accepted for treatment in this hospital from 8 May to 20 August 2003.

In this study, all SARS patients were diagnosed by the Beijing Center of Disease Control and Prevention according to the provisional clinical diagnostic criteria for SARS issued by the Chinese Ministry of Health on 15 April 2003 and were later confirmed by positive detection of serum antibodies to SARS-coronavirus.

SARS patients were divided into three groups. In the SARS group, patients were sampled 3 to 7 days after the onset of symptoms, and seven patients accepted steroid treatment for 1 to 2 days (methylprednisolone, 40 to 160 mg/day). In the severe SARS group, patients were sampled in the intensive care unit, and 18 patients received steroid treatment for 1 to 3 days (methylprednisolone, 80 to 160 mg/day). In the convalescent SARS group, patients were sampled 5 to 6 weeks after the onset of the disease, when the main signs and symptoms had disappeared; in addition there was air space opacity as determined by chest X-ray examination. Twenty-eight patients received steroid treatment (80 to 240 mg of methylprednisolone per day for 6 to 20 days before blood sampling). In the control group, blood samples were collected from volunteers that included doctors and nurses of the China-Japan Friendship Hospital, before they entered SARS areas. The basic patient information is summarized in Table 1.

Serum collection, preservation, and inactivation. All blood samples were collected under sterile conditions and without anticoagulant. Sera were collected and stored in a -80°C freezer after centrifugation at $800 \times g$ and 4°C for 10 min. All serum samples were inactivated by ^{60}Co gamma irradiation (2×10^6 rads) on dry ice before cytokine measurement (7).

Cytokine measurement by ELISA. Serum concentrations of IL-1 α , IL-4, IL-6, IL-8, IL-10, TGF- β , TNF- α , and IFN- γ were measured by ELISA by using Quantikine kits (R&D, Minneapolis, Minn.). The operating procedure provided by the manufacturer was strictly followed. The washing steps for microplates were carried out by using a programmable automatic washer (Athnos fluido; Athnos, Salzburg, Austria). The optical density at 450 nm was measured with an ELISA reader (Athnos 2010; Athnos). The minimum detectable concentration of all cytokines with the R&D kits was typically less than 10 pg/ml.

Statistical analysis. All results are expressed below as means \pm standard deviations. The unpaired Student *t* test was used to evaluate the differences between groups.

RESULTS

Serum TNF- α and IL-1 α levels did not change in SARS patients. The TNF- α concentrations in the control, SARS, severe SARS, and convalescent SARS groups were 59.6 ± 5.2 , 60.1 ± 4.4 , 57.8 ± 5.7 , and 61.9 ± 7.4 pg/ml, respectively. There were no significant differences between groups. The IL-1 α concentrations in the control, SARS, severe SARS, and convalescent SARS groups were 3.72 ± 0.56 , 3.80 ± 0.59 , 3.62 ± 0.39 , and 3.81 ± 0.33 pg/ml, respectively. The levels in the three SARS groups did not differ significantly from the levels in the control group (Fig. 1).

Serum IL-6 levels were increased in SARS patients. The IL-6 concentrations were 61.0 ± 10.1 , 163 ± 153 , 517 ± 796 , and 68.8 ± 25.9 pg/ml in the control, SARS, severe SARS, and convalescent SARS groups, respectively. Compared with the control subjects, statistically significant differences were found for the SARS and severe SARS patients but not for the convalescent SARS patients, suggesting that there was a positive relationship between the IL-6 concentration and the severity of SARS. The IFN- γ concentrations were 70.0 ± 20.4 , 63.0 ± 20.4 , 86.5 ± 41.1 , and 39.7 ± 4.9 pg/ml in the control, SARS, severe SARS, and convalescent SARS groups, respectively. The levels in the SARS and severe SARS groups were not significantly different from the levels in the control group, as shown in Fig. 2.

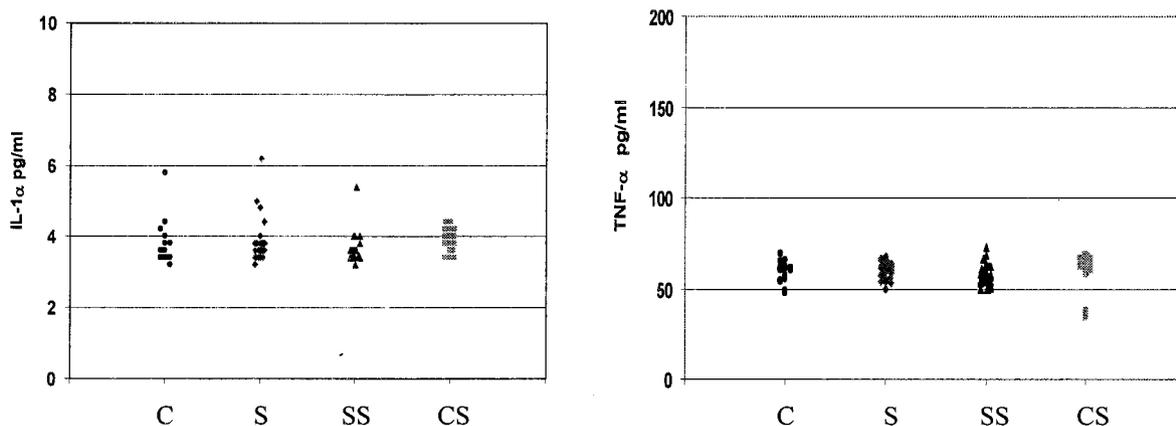


FIG. 1. Analysis of TNF- α and IL-1 levels in sera of SARS patients. C, control group; S, SARS group; SS, severe SARS group; CS, convalescent SARS group.

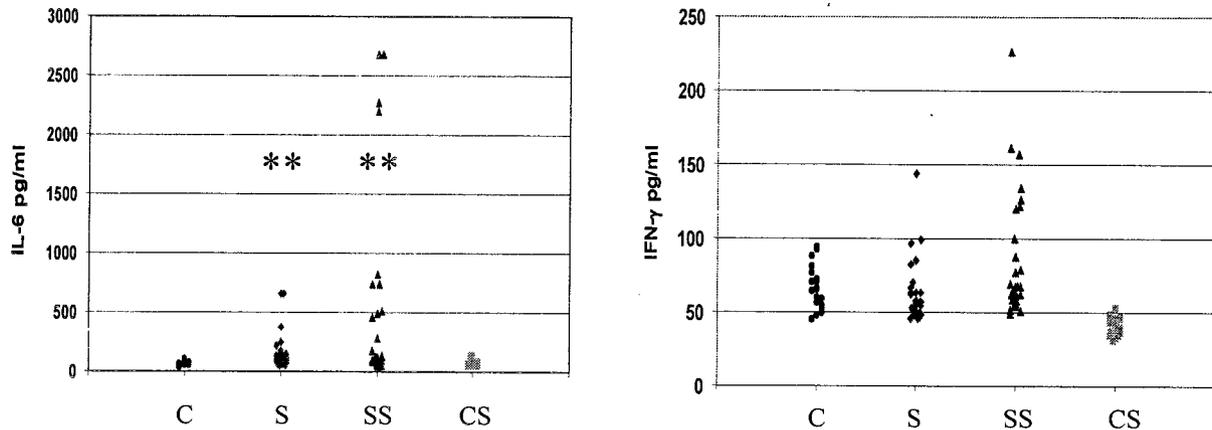


FIG. 2. Analysis of IL-6 and IFN- γ levels in sera of SARS patients. C, control group; S, SARS group; SS, severe SARS group; CS, convalescent SARS group. Two asterisks indicate that the P value is <0.01 .

Serum TGF- β and IL-8 levels were decreased in SARS patients. The IL-8 concentrations were 245 ± 72 , 165 ± 51 , 143 ± 41 , and 269 ± 180 pg/ml in the control, SARS, severe SARS, and convalescent SARS groups, respectively. The TGF- β concentrations were 48.7 ± 12.3 , 38.2 ± 15.2 , 37.3 ± 19.1 , and 55.5 ± 13.3 ng/ml, respectively, in the same groups. Compared with the control group, statistically significant differences for both of these cytokines were found for the SARS patients and the severe SARS patients but not for the convalescent SARS patients, suggesting that there was a negative relationship between the concentrations of IL-8 and TGF- β and SARS severity, as shown in Fig. 3.

Serum IL-4 and IL-10 levels were changed only in convalescent SARS patients. The IL-4 concentrations were 101 ± 22 , 109 ± 13 , 110 ± 12 , and 88 ± 8 pg/ml in the control, SARS, severe SARS, and convalescent SARS groups, respectively. The IL-10 concentrations were 48.8 ± 5.8 , 47.0 ± 5.3 , 49.7 ± 12.3 , and 169 ± 104 pg/ml, respectively, in the same groups. Compared with the control group, significant differences in the levels of the two cytokines were found in the convalescent

SARS patients but not in the patients with SARS or severe SARS, as shown in Fig. 4. The results for the eight cytokines studied in the sera of SARS patients are summarized in Table 2.

Effect of steroid treatment on serum cytokines. In order to evaluate the effect of steroid treatment on the serum cytokine levels in SARS patients, the levels of eight cytokines in patients who accepted corticosterone treatment were compared with the levels in patients who did not accept treatment with methylprednisolone in the SARS and severe SARS groups. No statistical differences between the two categories of patients were found for any of the cytokines tested, either in the SARS group or in the severe SARS group, as shown in Fig. 5.

DISCUSSION

In response to pathogens, host immune cells exhibit different reactions according to the nature of the infectious agent. The activation of inflammatory and immune systems is accompanied by cytokine release. Therefore, analysis of serum levels of

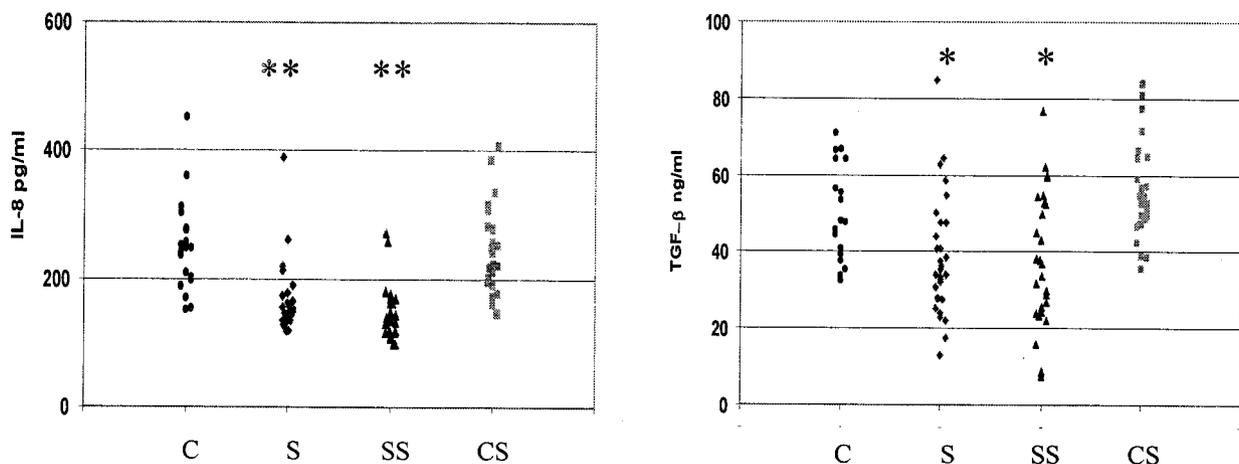


FIG. 3. Analysis of IL-8 and TGF- β levels in sera of SARS patients. C, control group; S, SARS group; SS, severe SARS group; CS, convalescent SARS group. Two asterisks indicate that the P value is <0.01 .

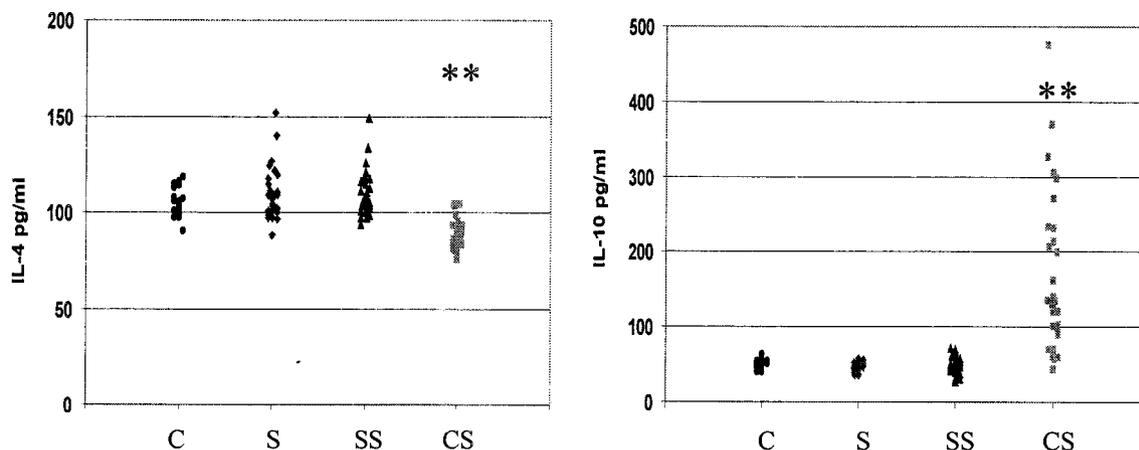


FIG. 4. Analysis of IL-4 and IL-10 levels in sera of SARS patients. C, control group; S, SARS group; SS, severe SARS group; CS, convalescent SARS group. Two asterisks indicate that the *P* value is <0.01.

cytokines may help us understand the functional alterations of the host immune system.

In this study, we analyzed serum cytokine levels in SARS patients and found four kind of changes. First, the serum TNF- α and IL-1 α concentrations did not differ significantly in SARS patients compared with control subjects. It has been reported that TNF- α and IL-1 play a key role in the pathogenesis of the acute lung injury in several infectious diseases (3). The level of TNF- α was also found to increase in some patients with a viral infection of the respiratory system, such as an influenza infection (12, 15). However, the levels of the two cytokines did not increase in the sera of SARS patients, suggesting that the host's immune reaction to the novel coronavirus might be different from the immune reaction to other pathogens. Although the serum cytokine concentrations may not reflect the levels of cytokines in injured lung tissue in some patients, the serum concentrations are likely to change if there are major changes in injured tissues (23). Therefore, analysis of cytokines in the bronchoalveolar lavage fluid of SARS patients should help us determine whether the cytokines participate in the pathology in injured lung tissue.

Second, the serum IL-6 level was increased in SARS patients. Compared with control subjects, a significant difference was found in patients with SARS or severe SARS but not in convalescent SARS patients, suggesting that there was a positive relationship between the IL-6 concentration and the severity of SARS. Although the serum IFN- γ concentration was

also increased in SARS patients, it was not significantly different from the concentration in control subjects, as shown in Fig. 2. IL-6 can be produced by either blood leukocytes or cells within the injured lung tissue, such as endothelial cells, fibroblasts, or alveolar epithelial cells (1, 11, 17, 22). Examination of the lung pathology of SARS showed that there were bronchial and alveolar epithelial necrosis and denudation (20). Electron microscopy revealed that particles of coronavirus were present in the cytoplasm of epithelial cells, suggesting that bronchial and alveolar epithelial cells are the main target cells of the virus (20). Therefore, we inferred that in SARS patients the increased serum IL-6 levels might originate from the injured pulmonary bronchial and alveolar epithelial cells. This hypothesis would be further confirmed if a higher concentration of IL-6 could be detected in bronchoalveolar lavage fluid from SARS patients.

Third, the concentrations of IL-8 and TGF- β in the sera were reduced in the SARS group, and the differences were significant in severe SARS patients but not in convalescent SARS patients, suggesting that the levels of both of these cytokines were negatively correlated with disease severity. In animal experiments, TGF- β has been shown to play an important role in acute lung injury, and it potentially could contribute to the development of pulmonary edema and pulmonary fibrosis (6, 8). Increased TGF- β levels were also found in the bronchoalveolar lavage fluid of patients with ARDS (2). Decreases in TGF- β levels have also been observed in some

TABLE 2. Serum cytokine analysis

Group	<i>n</i>	Concn of cytokines ^a							
		TNF- α (pg/ml)	IL-1 α (pg/ml)	IL-6 (pg/ml)	INF- γ (pg/ml)	IL-8 (pg/ml)	TGF- β (ng/ml)	IL-4 (pg/ml)	IL-10 (pg/ml)
Control	20	59.6 \pm 5.2	3.72 \pm 0.56	61 \pm 10.1	70 \pm 20.4	245 \pm 72	48.7 \pm 12.3	101 \pm 22	48.8 \pm 5.8
SARS	30	60.1 \pm 4.4	3.80 \pm 0.59	163 \pm 153 ^b	63.0 \pm 20.4	165 \pm 51 ^b	38.2 \pm 15.2 ^c	109 \pm 13	47.0 \pm 5.3
Severe SARS	30	57.8 \pm 5.7	3.62 \pm 0.39	517 \pm 796 ^b	86.5 \pm 41.1	143 \pm 41 ^b	37.3 \pm 19.1 ^c	110 \pm 12	49.7 \pm 12.3
Convalescent SARS	30	61.9 \pm 7.4	3.81 \pm 0.33	68.8 \pm 25.9	39.7 \pm 4.9 ^b	269 \pm 180	55.5 \pm 13.3	88 \pm 8 ^b	169 \pm 104 ^b

^a The data are means \pm standard deviations.

^b *P* < 0.01 for a comparison with the control group.

^c *P* < 0.05 for a comparison with the control group.

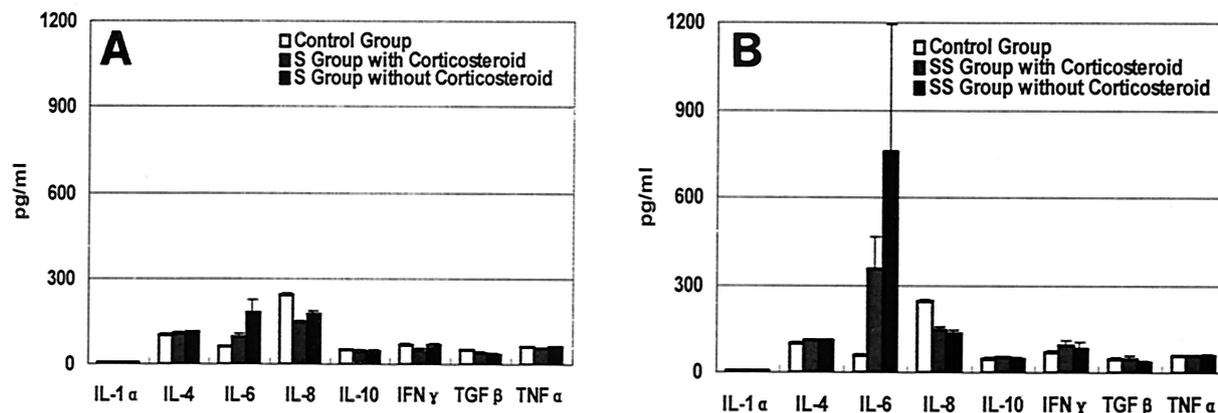


FIG. 5. Effect of steroid treatment on serum cytokines. (A) Control group ($n = 20$), SARS group (S Group) without methylprednisolone treatment ($n = 23$), and SARS group with methylprednisolone treatment ($n = 7$). (B) Control group ($n = 20$), severe SARS group (SS Group) without methylprednisolone treatment ($n = 12$), and severe SARS group with methylprednisolone treatment ($n = 18$). The results are expressed as means and standard errors.

patients with leukemia (4) or with severe AIDS dementia (21). The clinical significance of the decrease in the TGF- β level in the sera of SARS patients is not known. It has been reported that the levels of T lymphocytes were decreased, especially for the CD4 and CD8 subset, in SARS patients at an early stage and that the degree of the decrease was associated with the severity of SARS (24), suggesting that T-lymphocyte-dependent immune responses may be suppressed. Therefore, the significantly decreased levels of IL-8 and TGF- β in SARS patients may be consistent with the finding that T lymphocytes are depleted in these patients. In most cases, changes in serum IL-8 levels accompanied changes in IL-6 levels. The opposite changes for IL-6 and IL-8 may suggest different cell origins.

Fourth, some T-helper 2 cytokines, such as IL-4 and IL-10, showed altered serum concentrations in convalescent SARS patients but not in patients with SARS at an early stage. Compared with the levels in control subjects or SARS patients, the IL-4 levels were decreased and the levels of IL-10 were increased. It has been demonstrated that IL-10 inhibits TNF- α production and neutrophil activation in lipopolysaccharide-induced acute lung injury, which leads to a reduction in lung tissue injury (14). Therefore, the increased serum IL-10 levels in convalescent SARS patients may reflect some protective mechanisms, but the significance of this needs to be studied further.

Acute lung injury was the major pathological characteristic of SARS, as well as the main cause of death in SARS patients. This lung injury might be induced directly by the SARS pathogen or indirectly by cytokines that are released by activated inflammatory and immune cells, such as TNF- α , IL-1, and IFN- γ (3). However, our results indicated that these cytokines are not present at higher concentrations in the sera of either SARS or severe SARS patients, suggesting that acute lung injury in these patients may not be induced by circulating cytokines. In order to evaluate this possibility, sera from SARS patients were cocultured with human alveolar epithelial cells or pulmonary microvascular endothelial cells, and no cytotoxicity was found in either cell type after 24 to 48 h of incubation as determined by the methylthiazolotetrazolium assay (unpub-

lished data). Recently, we and another group found that some proteins of coronavirus exhibited toxic activity for human pulmonary endothelial cells and epithelial cells (unpublished data), and the relevant molecular mechanisms are being investigated.

Since some SARS patients had accepted steroid treatment when blood samples were collected, the potential effect of the steroids on serum cytokine levels needed to be investigated. Patients in the SARS and severe SARS groups were divided into two categories, one containing patients who were treated and one containing patients who were not treated. Our results show that there were not significant differences for the levels of any cytokine between these two categories (Fig. 5). This suggested that the short treatment did not affect the circulating levels of cytokines in these SARS patients. These results are consistent with the report that only long-term treatment (7 to 10 days) with a steroid can alter serum cytokine levels (19).

In conclusion, the changes in cytokines in the sera of SARS patients indicated that the reaction of the host's inflammatory and immune systems to the novel coronavirus is different from what is seen with other pathogens. The serum concentrations of IL-6, IL-8, and TGF- β may reflect the severity of SARS. These results suggest that there are different immunoregulatory events during and after SARS and may contribute to our understanding of the pathogenesis of this syndrome.

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