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ORIGINAL ARTICLE

# Elevated levels of circulating adhesion molecules in patients with active pulmonary tuberculosis

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**Elevated levels of circulating adhesion molecules in patients with active pulmonary tuberculosis**
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**Objective:** Recent studies have indicated the importance of cell adhesion molecules in the pathogenesis of various inflammatory lung diseases. Our study was designed to determine whether five soluble adhesion molecules including soluble L-, E- and P-selectin (sL-, sE- and sP-selectin), intercellular adhesion molecule-1 (sICAM-1), and vascular cell adhesion molecule-1 (sVCAM-1) in serum reflect the severity of active pulmonary tuberculosis (TB), and whether there is a distinct profile of these soluble molecules in this disease.

**Methodology:** Using enzyme-linked immunosorbent assays, we measured the serum levels of these five soluble adhesion molecules in 31 patients with active TB and 11 healthy volunteers.

**Results:** Serum levels of sE-selectin, sP-selectin and sICAM-1, but not sL-selectin or sVCAM-1, were significantly higher in patients with active TB than in the control subjects ($P < 0.001$, each). Significant correlations were detected only between serum levels of sE-selectin and sP-selectin, sE-selectin and sICAM-1, and sP-selectin and sICAM-1. There was a significant correlation between the Gaffky scale result (a scale assessing the number of mycobacteria bacilli present) and all of the above adhesion molecules, except for sL-selectin. Serum levels of sE-selectin, sL-selectin and sICAM-1 also correlated with the CXR radiological score. Higher levels of sL-selectin and sICAM-1 were detected in the serum of patients with radiological cavity formation compared to those without. The ESR, C-reactive protein and circulating neutrophil counts all correlated significantly with sE-selectin, sP-selectin, sICAM-1 and sVCAM-1.

**Conclusion:** The results suggest that there is a distinct profile of soluble adhesion molecules in active pulmonary TB and that sE-selectin, sP-selectin, and especially sICAM-1 appear to be the most sensitive clinical measures of disease severity.

**Key words:** pulmonary tuberculosis, soluble adhesion molecules.

## INTRODUCTION

Interactions between macrophages and T lymphocytes at inflammatory sites play a key role in the host defence against *Mycobacterium tuberculosis*.[1] Neutrophils also contribute to the control of *M. tuberculosis in vivo*,[2] while previous studies have demonstrated that lymphocyte alveolitis is present in patients with pulmonary tuberculosis (TB). BAL in these patients demonstrated increased numbers of activated macrophages, lymphocytes and neutrophils.[2–5] The mechanism by which these inflammatory cells accumulate in the lung is unknown at present, but it is likely that a selective and temporal expression of adhesion molecules plays a crucial role.[6–8]

Leukocytes migrate from the circulation into the inflamed tissue in a series of steps which includes their attachment to the endothelial wall followed by firm adhesion.[6–8] The selectin family of adhesion molecules and their respective ligands are important in the early transient 'rolling' phase of cell migration. The selectin family consists of three distinct carbohy-

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drate receptors expressed by leukocytes (L-selectin), endothelial cells (E-selectin), or platelets and endothelium (P-selectin).[8] Both intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are members of the immunoglobulin supergene family. ICAM-1 acts as a ligand for the β2 leukocyte integrins, such as lymphocyte function-associated antigen (LFA)-1 and Mac-1,[9,10] while VCAM-1 binds the very late antigen (VLA)-4, one of the β1 integrins.[11] A number of cell adhesion molecules have been detected as soluble circulating forms in human serum and other body fluids, and elevated levels of these soluble forms have been found in numerous lung diseases.[12–19] It is likely that these soluble adhesion molecules originate from adhesion molecules expressed on activated cells, and that their presence in the circulation may reflect the severity and activity of inflammation in pulmonary TB. In support of this, high concentrations of soluble ICAM-1 have been detected in the sera of patients with TB compared with normal controls.[15,16]

In the present study, we measured the serum levels of sL-selectin, sE-selectin, sP-selectin, sICAM-1, and sVCAM-1 in patients with active pulmonary TB. We determined whether serum levels of these adhesion molecules correlated with disease severity, and if a specific profile occurs in active pulmonary TB.

## MATERIALS AND METHODS

### Study population

We studied 31 consecutive patients with active pulmonary TB who were admitted to Miyazaki Medical College and National Sanatorium Miyazakihigashi Hospital, Miyazaki, Japan, between July 1998 and November 1999. They consisted of 11 women and 20 men, aged 50.7 ± 19.2 years. Active pulmonary TB was diagnosed by emergence of new shadows on CXR, clinical features, and at least one positive sputum smear for acid-fast bacilli or a positive sputum culture for *M. tuberculosis*. Patients with a history of other lung diseases, recurrent pulmonary TB, miliary TB, extrathoracic TB, or coinfection with HIV were excluded from this study. No patient had any other systemic disease, and none had been treated with anti-TB therapy in the past. We also selected 11 healthy volunteers (four women and seven men, aged 33.3 ± 3.0 years) as controls. All had normal CXR, were free of symptoms, and were not taking any medications at the time of recruitment. The study protocol was approved by the Human Ethics Review Committees of Miyazaki Medical College and a signed consent form was obtained from each subject.

### CXR and CT scans

Before commencement of anti-TB treatment, a CXR and CT scan were obtained for all patients. Patients were divided into two groups based upon the presence or absence of a radiological cavity on their CT scan. In addition, the CXR findings were scored according to the extent of lesions using the recommendations of the Japan Society of Chemotherapy.

The following scoring was used: 0, no abnormal shadows; 1, shadows over less than one intercostal space; 2, shadows involving an area between 1 and 3; 3, shadows involving one-tenth of a hemi-thorax; 4, shadows involving an area between 3 and 5; 5, shadows involving one-third of a hemi-thorax; 6, shadows involving an area between 5 and 7; 7, shadows involving two-thirds of a hemi-thorax; 8, shadows in an entire hemi-thorax; 9, shadows involving an area between 8 and 10; and 10, shadows extensively involving both lungs.

### Immunoassay

The serum levels of C-reactive protein (CRP) were measured using a commercial assay kit (Denka Seiken Co. Ltd., Tokyo, Japan). The serum levels of soluble L-, E-, and P-selectin (sL-, sE- and sP-selectin), ICAM-1 (sICAM-1) and VCAM-1 (sVCAM-1) were measured using commercial assay kits (R & D Systems Europe, Abingdon, UK). The serum samples were diluted 1 : 20 for sE-, sP-selectin and sICAM-1, 1 : 50 for sVCAM-1 and 1 : 100 for sL-selectin assays before the assay was performed. Enzyme-linked immunosorbent assays (ELISA) were used. They were standardized against purified forms of recombinant soluble molecules. Plates were read at 450 nm in an ELISA reader. Duplicate assays were performed for serial dilutions of each sample and the average value was recorded. The sensitivities of these assays were 9.0 ng/mL, 0.4 ng/mL, 4.6 ng/mL, 2.8 ng/mL, and 140 ng/mL for sL-, E- and P-selectin, sICAM-1 and sVCAM-1, respectively. The intra- and interassay coefficients of variation were 4.1% and 7.1% for sL-selectin, 4.7% and 7.4% for sE-selectin, 5.6% and 7.9% for sP-selectin, 4.6% and 6.1% for sICAM-1, and 4.9% and 8.9% for sVCAM-1.

### Statistical analysis

Data were expressed as mean ± standard deviation (SD). The Mann–Whitney *U*-test was used for between-group comparisons. The correlation between two parameters was examined by Spearman's rank correlation test. A *P*-value of less than 5% was considered statistically significant.

## RESULTS

The mean serum concentrations of the panel of five soluble adhesion molecules in patients with active pulmonary TB are shown in Figs 1 and 2. The serum levels of sE- and sP-selectin and ICAM-1 were significantly elevated in patients with TB compared with control subjects, but there were no differences in the levels of sL-selectin and sVCAM-1 between the two groups. Comparison of the serum concentration of each soluble cell adhesion molecule with the level of the other molecules in both healthy subjects and

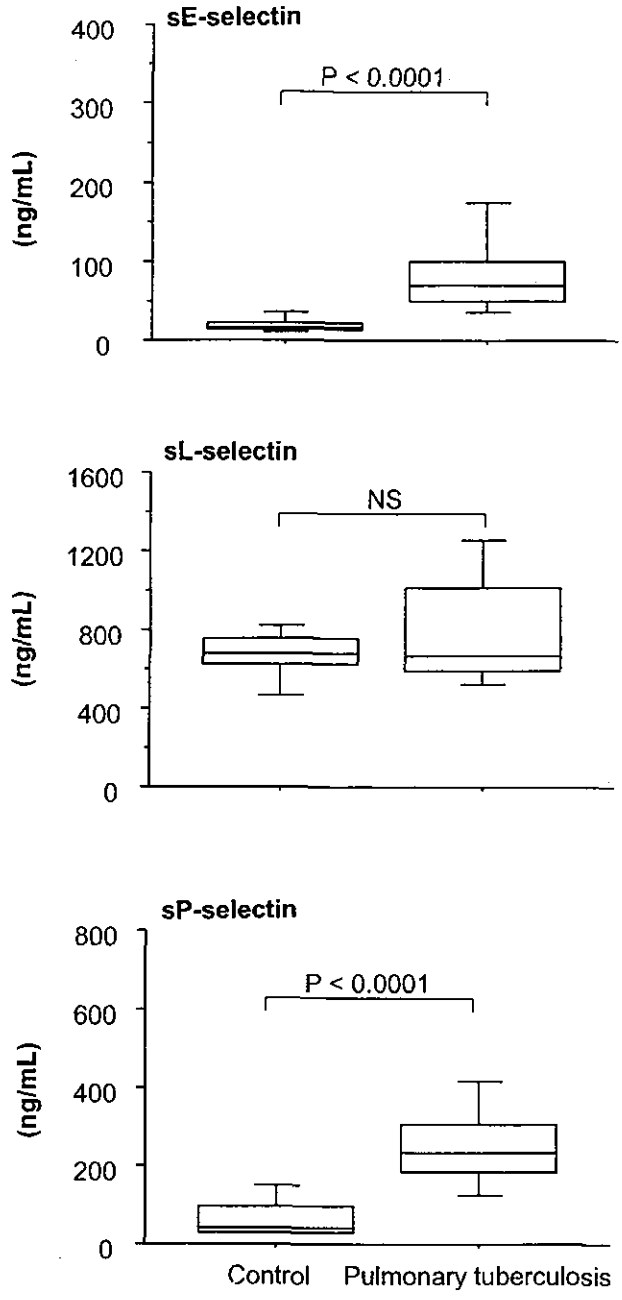


**Figure 1** Serum levels of soluble E-, L- and P-selectin in healthy subjects ($n=11$) and patients with active TB ($n=31$). The whisker box plots represent the 25th to 75th percentiles of values. The median is indicated by the horizontal bar inside the box, and the whiskers on each box represent the 10th and 90th percentiles. Control, healthy subjects; NS, not significant.

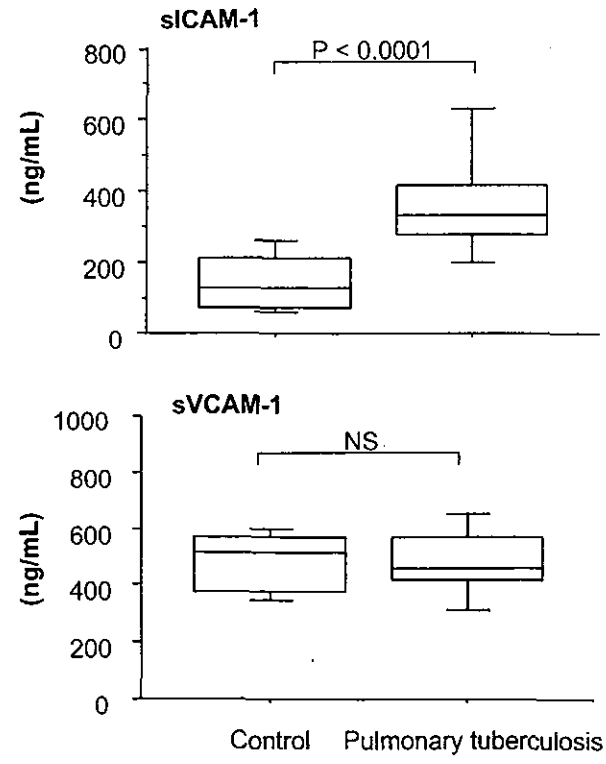


**Figure 2** Serum levels of soluble intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in healthy subjects ($n=11$) and patients with active TB ($n=31$). The whisker box plots represent the 25th to 75th percentile of results inside the box. The median is indicated by the horizontal bar inside the box, and the whiskers on each box represent the 10th and 90th percentiles. Control, healthy subjects; NS, not significant.

patients with active TB demonstrated significant positive correlations between the levels of sE-selectin and sP-selectin, sE-selectin and sICAM-1, and sP-selectin and sICAM-1 (Table 1).

A correlation analysis between the levels of these soluble adhesion molecules and the Gaffky scale, a scale used to denote the prognosis in TB based on the number of tubercle bacilli in sputum from patients with TB, revealed significant correlations for all soluble molecules except sL-selectin (Table 2). The serum concentrations of sE-selectin, sL-selectin and sICAM-1 also correlated with the radiological score (see Materials and methods section) (Table 2). Of the 31 patients, 11 patients showed cavity formation on the CXR and CT scan. Higher concentrations of sL-selectin and sICAM-1 were detected in the serum of patients with cavity formation than those without a cavity (Table 3). In addition, the ESR, the serum CRP level and the total peripheral blood leukocyte and neutrophil counts each correlated significantly with sE-selectin, sP-selectin, sICAM-1, and sVCAM-1 concentration (Table 4). However, there was no significant correlation between these adhesion molecules and pulmonary function tests (VC % and $FEV_1$%) in patients with TB (data not shown).

## DISCUSSION

In active TB there appears to be a distinct pattern of elevated serum levels of adhesion receptors that correlates with disease severity. The serum concentrations of sE-selectin, sP-selectin, and sICAM-1, but not sL-selectin or sVCAM-1 were significantly increased

**Table 1** Correlation between levels of soluble adhesion molecules in controls and patients with pulmonary tuberculosis

| | sE-selectin | sL-selectin | sP-selectin | sICAM-1 | sVCAM-1 |
|---|---|---|---|---|---|
| sE-selectin | | | | | |
| sL-selectin | NS | | | | |
| sP-selectin | $r = 0.77$ ($P < 0.001$) | NS | | | |
| sICAM-1 | $r = 0.78$ ($P < 0.001$) | NS | $r = 0.62$ ($P < 0.001$) | | |
| sVCAM-1 | NS | NS | NS | NS | |

sL-, sE- and sP-selectin, soluble L-, E- and P-selectin; sICAM-1, intercellular adhesion molecule-1; sVCAM-1, vascular cell adhesion molecule-1; NS, not significant.

**Table 2** Correlation between levels of soluble adhesion molecules and Gaffky number or radiological findings in pulmonary tuberculosis

| | Gaffky number | Radiological score |
|---|---|---|
| sE-selectin | $r = 0.46$ ($P = 0.011$) | $r = 0.39$ ($P = 0.034$) |
| sL-selectin | NS | $r = 0.46$ ($P = 0.012$) |
| sP-selectin | $r = 0.41$ ($P = 0.025$) | NS |
| sICAM-1 | $r = 0.54$ ($P = 0.003$) | $r = 0.64$ ($P = 0.001$) |
| sVCAM-1 | $r = 0.42$ ($P = 0.020$) | NS |

sL-, sE- and sP-selectin, soluble L-, E- and P-selectin; sICAM-1, intercellular adhesion molecule-1; sVCAM-1, vascular cell adhesion molecule-1; NS, not significant.

in active TB patients. More importantly, the serum levels of sE-selectin, and sP-selectin, and especially sICAM-1 correlated well with disease severity including measures such as the Gaffky number, CXR scores and levels of inflammatory markers (ESR, CRP, and leukocyte and neutrophil counts in the peripheral blood).

Previous studies have assessed the level of one or more soluble adhesion molecules in TB.[15,16] Shijubo *et al.* found elevated levels of sICAM-1 in miliary and advanced TB, but not in minimal or moderately advanced disease.[15] Another study demonstrated that sICAM-1 and sE-selectin levels were elevated in patients with active TB, compared with treated patients and healthy controls.[16] However, these previous reports did not examine the relationship between these molecules and disease severity. The present data confirm and expand these previous findings.

ICAM-1 is an adhesion molecule expressed on cells of multiple lineages, and is upregulated at sites of inflammation, while E-selectin is expressed only on the surface of activated endothelial cells.[6-8] *In vitro* studies have shown that antibodies to ICAM-1 inhibit leukocyte adhesion to endothelial cells and granulocyte migration through the endothelium,[20] and *in vivo* studies in rabbits confirmed that antibodies to ICAM-1 inhibit neutrophil trafficking into inflamed lungs.[21] Furthermore, antibodies directed against E-selectin block neutrophil extravasation and vascular leakage in rat lungs.[22] Both ICAM-1 and E-selectin molecules are shed from endothelial cells after exposure to mediators such as tumour necrosis factor (TNF)-α and IL-1.[23] The increase in serum levels and the significant correlations between circulating sICAM-1 and sE-selectin in our patients is likely to reflect the upregulation of these adhesion molecules on vascular endothelial cells in lung tissue. We have demonstrated previously that circulating soluble adhesion molecules might correlate with cytokines produced in the lungs of patients with diffuse panbronchiolitis.[14] In BAL fluid of patients with active TB increased levels of several cytokines including IL-1, IL-6 and TNF-α have been reported.[24-26] Such cytokines might cause local activation of endothelial cells leading to increased expression and release of ICAM-1 and E-selectin.

In the present study, serum concentrations of sICAM-1 reflected disease severity in comparison with sE-selectin. In this context, Lopez Ramirez and colleagues reported that exposure of a monocyte/macrophage cell line to *M. tuberculosis* elicited a sustained increase in the expression of ICAM-1, but not VCAM-1 or LFA-1.[27] Furthermore, Rothlein and coworkers have shown that peripheral blood mononuclear cells release detectable levels of sICAM-1 in culture.[28] Thus, ICAM-1 is expressed on a variety of cells in the lung and in the circulation. The source of the sICAM-1 detected in the serum of TB patients remains unclear, but we speculate that it may be derived from other cells in addition to activated endothelial cells.

In the present study, we observed a differential rise in sICAM-1 and sE-selectin, but not sVCAM-1. This finding seems physiologically compatible, as VCAM-1 is involved in binding monocytes, lymphocytes and eosinophils, but not neutrophils, to activated endothelium[29] and neutrophils play an important role in the host response in active and advanced TB.[2] However, our finding is not in agreement with a report by Lai *et al.*[16] who demonstrated a significant elevation of sVCAM-1 in both active and old TB compared with controls. Feng and colleagues demonstrated that upregulation of VCAM-1 is associated with immunity to pulmonary *M. tuberculosis* infection in mice.[30] This discrepancy might be explained, at least in part, by differences in the study populations with regard to species, race, disease stage, and activity. Nevertheless, our findings did demonstrate that the levels of sVCAM-1 correlated weakly but significantly with the Gaffky number, ESR, CRP, and the number of white blood cells and neutrophils in peripheral blood, suggesting that VCAM-1 might also be playing a role in

**Table 3** Cavity formation and levels of soluble adhesion molecules

| Cavity formation | sE-selectin | sL-selectin | sP-selectin | sICAM-1 | sVCAM-1 |
|---|---|---|---|---|---|
| (+) | 76.2 | 980.8 | 284.4 | 399.1 | 498.1 |
| | (43.0–414.0) | (439.2–1254.9) | (139.4–672.6) | (277.7–836.6) | (334.7–945.0) |
| (–) | 66.0 | 610.3 | 233.2 | 315.4 | 454.3 |
| | (24.0–210.7) | (312.5–1477.4) | (99.5–508.5) | (64.5–662.6) | (280.0–834.5) |
| | NS | $P < 0.05$ | NS | $P < 0.05$ | NS |

sL-, sE- and sP-selectin, soluble L-, E- and P-selectin; sICAM-1, intercellular adhesion molecule-1; sVCAM-1, vascular cell adhesion molecule-1; NS, not significant.
Data are median (range).

**Table 4** Correlation between levels of soluble adhesion molecules and inflammatory markers

| | Erythrocyte sediment rate | C-reactive protein | White blood cell count | No. neutrophils |
|---|---|---|---|---|
| sE-selectin | $r = 0.46$ ($P = 0.011$) | $r = 0.37$ ($P = 0.044$) | $r = 0.60$ ($P = 0.001$) | $r = 0.66$ ($P = 0.001$) |
| sL-selectin | NS | NS | NS | NS |
| sP-selectin | $r = 0.41$ ($P = 0.025$) | $r = 0.42$ ($P = 0.022$) | NS | $r = 0.44$ ($P = 0.016$) |
| sICAM-1 | $r = 0.54$ ($P = 0.003$) | $r = 0.61$ ($P = 0.001$) | $r = 0.65$ ($P = 0.001$) | $r = 0.66$ ($P = 0.001$) |
| sVCAM-1 | $r = 0.42$ ($P = 0.020$) | $r = 0.37$ ($P = 0.044$) | $r = 0.39$ ($P = 0.033$) | $r = 0.48$ ($P = 0.008$) |

sL-, sE- and sP-selectin, soluble L-, E- and P-selectin; sICAM-1, intercellular adhesion molecule-1; sVCAM-1, vascular cell adhesion molecule-1; NS, not significant.

the generation of an effective pulmonary immune response.

L- and P-selectins also mediate the initial phase of the adhesion cascade, involving leukocyte rolling. Previous reports have suggested that soluble forms of these selectins in the circulation serve as useful indicators of the severity of acute lung injury.[31] We have previously demonstrated the possible role of L-selectin in T lymphocyte alveolitis in patients with active pulmonary sarcoidosis[19] and that the serum levels of selectins, ICAM-1 and VCAM-1 are increased in diffuse panbronchiolitis.[14] Littler and colleagues have demonstrated that of six soluble adhesion molecules, only sP-selectin correlated with disease activity in rheumatoid arthritis.[32] These results all suggest that these selectins may participate in the pathogenesis of active pulmonary TB, albeit that the serum levels of sL-selectin in TB were similar to those in controls. Although we cannot explain this particular finding, it is possible that sL-selectin does not reflect disease severity in active TB.

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# Relationship between whole-blood interferon-gamma production and extent of radiographic disease in patients with pulmonary tuberculosis

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**Abstract**

The aim of this study was to determine whether whole-blood interferon-γ (IFN-γ) production correlates with the radiographic extent of pulmonary tuberculosis before treatment. The subjects were 40 human immunodeficiency virus-negative patients with pulmonary tuberculosis and 36 healthy volunteers. The concentrations of IFN-γ in whole blood stimulated with *Mycobactrium tuberculosis* purified protein derivatives (tuberculous PPD) and phytohemagglutinin (PHA) were evaluated. PHA-stimulated IFN-γ (PHA-IFN-γ) was lower in the patients than in healthy volunteers ($p < 0.05$), and inversely correlated with the disease extent ($p < 0.01$). Tuberculous PPD-stimulated IFN-γ as a percentage of PHA response (tuberculous PPD-IFN-γ/PHA-IFN-γ) was higher in the patients than in healthy volunteers ($p < 0.05$). However, tuberculous PPD-IFN-γ/PHA-IFN-γ did not correlate with the disease extent. Our results indicate that the tuberculous PPD-IFN-γ/PHA-IFN-γ may be useful for the diagnosis of tuberculosis but not for evaluating the disease severity, and suggest that PHA-IFN-γ could be considered as a marker of disease severity. 

*Keywords:* Laboratory diagnosis; Tuberculosis; Interferon-γ; Radiography

## 1. Introduction

Pulmonary tuberculosis is one of the most important infectious diseases in the world. Both tuberculosis occurring in immunodeficient patients and multidrug-resistant tuberculosis have emerged as clinical problems. The effects of conventional anti-tuberculosis drugs are limited in such clinical settings, and new treatment strategies are needed (Pozniak, 2001). The cellular immune response is closely involved in the presentation and progression of tuberculosis (Schluger and Rom, 1998). For the prevention and treatment of refractory tuberculosis, the immune response must be evaluated in each patient. To date, the immune response to *Mycobactrium tuberculosis* has been assessed by skin testing using the purified protein derivative from *M. tuberculosis* (tuberculous PPD). In recent years, the whole-blood interferon-γ (IFN-γ) assay has been anticipated to become a method for early diagnosis of tuberculosis infection (Andersen et al., 2000; Mazurek et al., 2001). This test measures IFN-γ release from whole blood cells stimulated with tuberculous PPD adjusted by the control mitogen, phytohemagglutinin (PHA). This assay has a sensitivity of 90% and a specificity of 98% for the diagnosis of tuberculosis, which are better than those of the tuberculin skin test (Streeton et al., 1998). Recently, a Food and Drug Administration panel has approved the whole-blood IFN-γ test for the diagnosis of latent tuberculosis infection (Center for Disease Control and Prevention, 2001). However, whether the results of the whole-blood IFN-γ assay correlate with disease severity is not clear at present. Although the importance of radiographic extent of tuberculosis is decreased after effective drugs are available, death due to tuberculosis is associated with the disease extent (Humphries et al., 1984; Nagayama et al., 2001). The present study was designed to determine whether the results of the whole-blood IFN-γ assay correlated with the radiographic extent of pulmonary tuberculosis before treatment. Our study demonstrated that the disease extent of pulmonary tuberculosis

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Table 1
Lymphocyte subsets in tuberculosis patients and healthy volunteers*

| Cell type | Healthy volunteers (n = 36) | | Tuberculosis patients (n = 40) | | P | Sex, age-adjusted P |
|---|---|---|---|---|---|---|
| | Median | 25th–75th | Median | 25th–75th | | |
| WBC, cells/μL | 5300 | 4185–6650 | 4975 | 4090–6830 | NS | NS |
| Lymphocyte, cells/μL | 1932 | 1575–2382 | 1309 | 914–1914 | <0.0001 | <0.05 |
| $CD3^+$, cells/μL | 1300 | 933–1534 | 668 | 510–1116 | <0.0001 | <0.05 |
| $CD4^+$, cells/μL | 842 | 636–1088 | 489 | 326–650 | <0.0001 | <0.05 |
| $CD8^+$, cells/μL | 476 | 404–593 | 336 | 221–505 | <0.05 | NS |
| $CD56^+$, cells/μL | 430 | 319–545 | 387 | 216–614 | NS | NS |

* Data are expressed as the median, 25th, and 75th percentile values.

was inversely correlated with PHA-stimulated IFN-γ production, but not with tuberculous PPD-stimulated IFN-γ as a percentage of the PHA response.

## 2. Patients and methods

### 2.1. Patients

The subjects were adult patients with newly diagnosed and culture-positive pulmonary tuberculosis. Patients who were receiving immunosuppressive therapy or who were positive for human immunodeficiency virus (HIV) were ineligible for the study. As controls, we selected BCG-vaccinated healthy volunteers without a past history of tuberculosis or other *Mycobacterium* infections. The volunteers received the repeated doses of BCG at infant. The healthy subjects were determined by history taking, physical examination, and chest radiography. All participants gave their informed consent prior to the study. Blood samples, sputum smear, and chest x-ray films were obtained before treatment for tuberculosis was administered.

### 2.2. Lymphocyte subsets

Blood samples for cell counts were placed into heparinized tubes. Total leukocyte counts and differential cell counts were determined with an automated hematology analyzer (NE-8000, Sysmex Corporation, Kobe, Japan). Lymphocyte subsets were evaluated using flow cytometry. Briefly, heparinized blood was incubated with fluorescein isothiocyanate-conjugated monoclonal antibody for CD3, CD4, CD8, and CD56 (BD Biosciences, Franklin Lakes, NJ). After lysis of erythrocytes, 10,000 cells were analyzed using the FACSCalibur system (BD Biosciences).

### 2.3. Whole blood IFN-γ production

Blood was collected into heparinized tubes. One-ml aliquots of blood, in a 24-well culture plate, were stimulated with three drops of either saline (background), tuberculous PPD, PPD from *Mycobactrium avium* complex (avian PPD) or PHA. The tuberculous PPD, avian PPD and PHA were obtained from CSL Limited (Parkville, Australia). After a 16-h incubation at 37°C in a humidified incubator, the supernatants were collected. The amount of IFN-γ in the supernatants was quantified by the enzyme-linked immunosorbent assay kit (Endogen Inc., Woburn, MA). Concentrations of IFN-γ released by the antigens were reported as the levels after subtracting the background release. The concentrations of PHA-stimulated IFN-γ, tuberculous PPD-stimulated IFN-γ, and avian PPD-stimulated IFN-γ were expressed as PHA-IFN-γ, tuberculous PPD-IFN-γ, and avian PPD-IFN-γ, respectively. The responses to tuberculous PPD and avian PPD were further evaluated as a percentage of the PHA response, which was expressed as

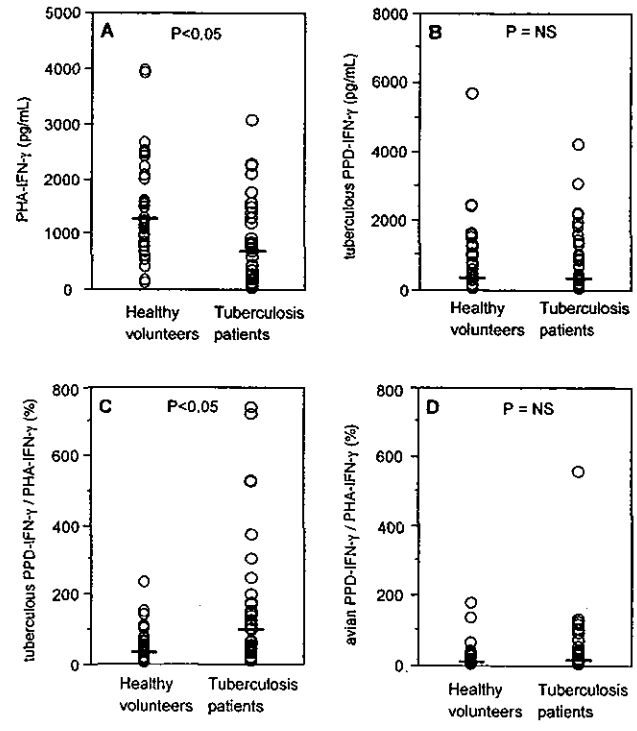


Fig. 1. IFN-γ production in tuberculosis patients and healthy volunteers. The horizontal bars represent the median values. A: PHA-stimulated IFN-γ. B: *M. tuberculosis* PPD-stimulated IFN-γ. C: *M. tuberculosis* PPD-stimulated IFN-γ as a percentage of PHA-stimulated IFN-γ. D: *M. avium* PPD-stimulated IFN-γ as a percentage of PHA-stimulated IFN-γ.

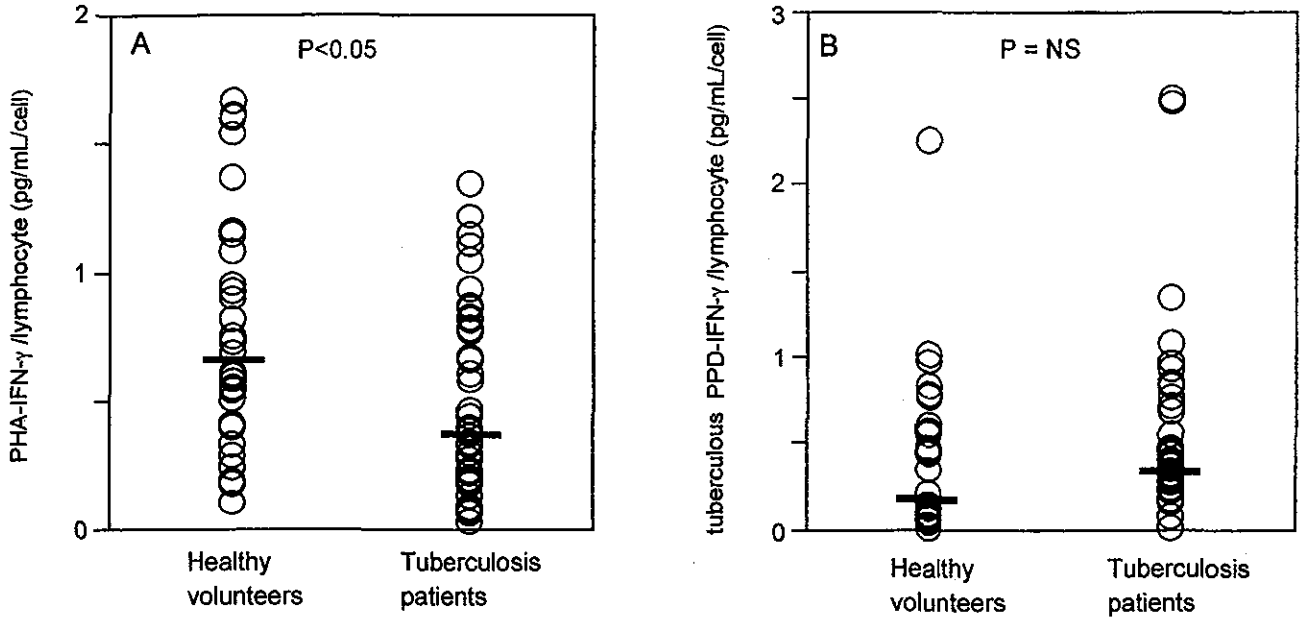


Fig. 2. IFN-γ production per lymphocyte in tuberculosis patients and healthy volunteers. The horizontal bars represent the median values. A: PHA-stimulated IFN-γ, B: *M. tuberculosis* PPD-stimulated IFN-γ.

tuberculous PPD-IFN-γ/PHA-IFN-γ and avian PPD-IFN-γ/PHA-IFN-γ, respectively.

### 2.4. Extent of pulmonary tuberculosis

The chest x-ray films were interpreted by two physicians. The radiographic extent of pulmonary tuberculosis was assessed according to the following classification (Kardjito and Grange, 1980): minimal disease (the total diseased area did not exceed the area above the second chondrosternal junction and the fourth thoracic vertebra); moderately advanced disease (dense and confluent lesions did not exceed an area of one-third of one lung field, or non-confluent lesions did not exceed the area of one lung); and far advanced disease (more extensive than moderately advanced disease).

### 2.5. Statistical analysis

The number of lymphocytes and the amount of IFN-γ were expressed as the median, 25th and 75th percentile values. Differences between tuberculosis patients and healthy volunteers were evaluated by the Mann-Whitney *U* test. Since healthy volunteers were not matched to patients by age and sex, the sex- and age-adjustment was retrospectively conducted by logistic regression analysis. The relationship of IFN-γ production with disease extent was assessed by Spearman correlation. Two-tailed $p < 0.05$ was considered significant. Data were analyzed with the StatView software program (version 5.0; SAS Institute Inc., Cary, NC).

## 3. Results

### 3.1. Patient characteristics

The enrolled subjects comprised 40 patients (28 males and 12 females) with pulmonary tuberculosis and 36 healthy volunteers (18 males and 18 females). The proportion of males in the patients was slightly higher than in healthy volunteers, but was not significantly different. The mean age (± standard deviation) of patients and healthy volunteers was 68 ± 17 and 49 ± 18 years, respectively. The patients were significantly older than healthy volunteers ($p < 0.0001$). With respect to underlying diseases, nine of 40 patients suffered from diabetes mellitus. Twenty-eight patients of 40 patients had acid-fast bacilli-positive sputum smears. The radiographic extent of pulmonary tuberculosis included 15 cases of minimal disease, 17 of moderately advanced disease, and eight of far advanced disease. The positive sputum smear was seven cases of minimal disease, 15 of moderately advanced disease, and six of far advanced disease. Two patients died of tuberculosis during the course of treatment: one patient with moderately advanced disease and the other patient with far advanced disease.

### 3.2. Lymphocyte subsets

The numbers of total lymphocytes, $CD3^+$, $CD4^+$ and $CD8^+$ T cells were lower in patients than in the control (Table 1). The median number of lymphocytes and $CD4^+$ cells in the patients was 1309/μL and 489/μL, respectively. Even after adjustment for sex and age, differences in the counts of total lymphocytes, $CD3^+$ and $CD4^+$ cells re-

mained significant between the two groups ($p < 0.05$, each). In contrast, the difference in $CD8^+$ cell numbers was not observed after adjustment for sex and age. The low $CD3^+$ cell count in patients was mainly due to a reduction in $CD4^+$ cells. The numbers of total lymphocytes, $CD3^+$ and $CD4^+$ cells did not correlate with the presence of diabetes mellitus or a positive sputum smear (data not shown).

### 3.3. Whole-blood IFN-γ production

PHA-IFN-γ was lower in patients than in normal subjects (Fig. 1; $p < 0.05$ after adjustment for sex and age). The median value of PHA-IFN-γ was 700 pg/mL in patients and 1280 pg/mL in healthy volunteers (Fig. 1). In contrast, there was no difference in tuberculous PPD-IFN-γ between patients and healthy volunteers. However, tuberculous PPD-IFN-γ/PHA-IFN-γ was higher in patients than in the control subjects (median, 100% *vs.* 35%; $p < 0.05$ after adjustment for sex and age), whereas avian PPD-IFN-γ/PHA-IFN-γ did not differ between the two groups. PHA-IFN-γ, tuberculous PPD-IFN-γ and tuberculous PPD-IFN-γ/PHA-IFN-γ did not correlate with the presence of diabetes mellitus or a positive sputum smear (data not shown).

Numbers of cultured lymphocytes were different in each sample. IFN-γ is known to be released from lymphocytes, particularly from $CD4^+$, $CD8^+$ and $CD56^+$ cells. Accordingly, we further evaluated IFN-γ production adjusted by the number of lymphocytes (Fig. 2). PHA-IFN-γ per lymphocyte remained lower in patients than in healthy volunteers (median, 0.47 *vs.* 0.65 pg/mL/cell; $p < 0.05$ after sex- and age-adjustment). Tuberculous PPD-IFN-γ per lymphocyte was not different between the two groups.

### 3.4. Relationship of disease extent and whole-blood IFN-γ production

Counts of lymphocytes, $CD3^+$ and $CD4^+$ cells correlated inversely with the severity of the disease, whereas the numbers of $CD8^+$ cells did not correlate with the disease extent (Fig. 3). PHA-IFN-γ and tuberculous PPD-IFN-γ correlated inversely with the disease extent (Fig. 4; $r = -0.53$, $p < 0.01$ and $r = -0.50$, $p < 0.01$, respectively). In addition, PHA-IFN-γ per lymphocyte and tuberculous PPD-IFN-γ per lymphocyte were also reduced in advanced pulmonary lesions (data not shown). In contrast, tuberculous PPD-IFN-γ/PHA-IFN-γ did not correlate with disease extent (Fig. 4).

## 4. Discussion

The present study demonstrated that although tuberculous PPD-IFN-γ/PHA-IFN-γ was increased in patients with tuberculosis, this parameter did not correlate with the disease extent. In contrast, advanced pulmonary disease was

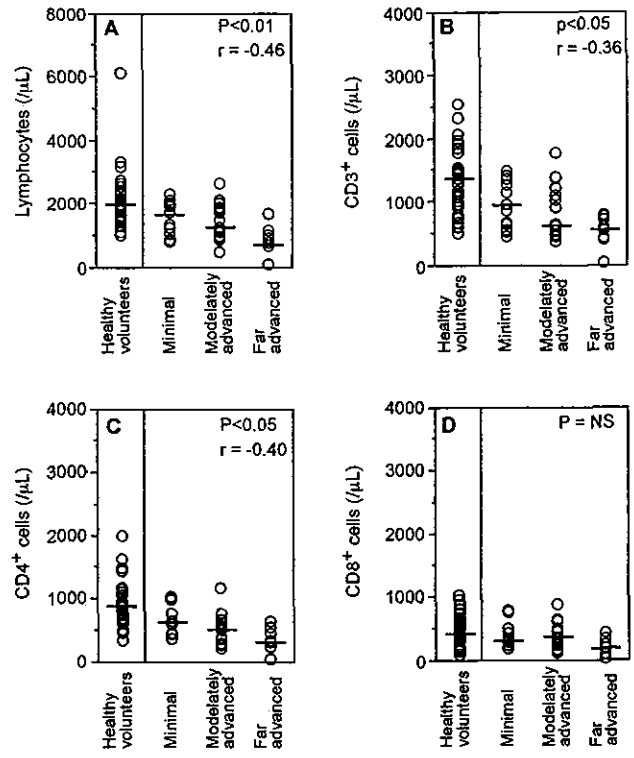


Fig. 3. Correlation between lymphocyte subsets and the radiographic extent of pulmonary tuberculosis. The horizontal bars represent the median values. Disease extent is expressed as minimal, moderately advanced or far advanced disease. The correlation was evaluated by the Spearman method, excluding the results of healthy subjects. A: total lymphocytes, B: $CD3^+$ cells, C: $CD4^+$ cells, D: $CD8^+$ cells.

associated with both a reduction of PHA-IFN-γ production and a depression of the lymphocyte count.

Both the number and function of lymphocytes can influence the amount of IFN-γ production in whole blood. In the present study, counts of lymphocytes and $CD4^+$ cells were depressed in patients with tuberculosis. Furthermore, PHA-IFN-γ production per lymphocyte as well as whole-blood PHA-IFN-γ production were both decreased in patients. Several investigators have also reported that counts of lymphocytes and $CD4^+$ cells are reduced in HIV-negative patients with tuberculosis (Jones et al., 1997). In addition, peripheral blood leukocytes that are stimulated with several mitogens, including PHA, produce less IFN-γ in patients with tuberculosis (Vilcek et al., 1986). These findings suggest that peripheral blood lymphocytes may be quantitatively and qualitatively depressed in HIV-negative patients with tuberculosis.

In patients with tuberculosis, unlike PHA-IFN-γ, tuberculous PPD-IFN-γ production was not always decreased, but rather, tuberculous PPD-IFN-γ/PHA-IFN-γ was increased. In contrast, avian PPD-IFN-γ/PHA-IFN-γ was not increased in the patients. Taken together, the high percentage of tuberculous PPD-IFN-γ/PHA-IFN-γ in patients was not a consequence of decreased PHA-IFN-γ. Our results differ from those of earlier studies, which showed that

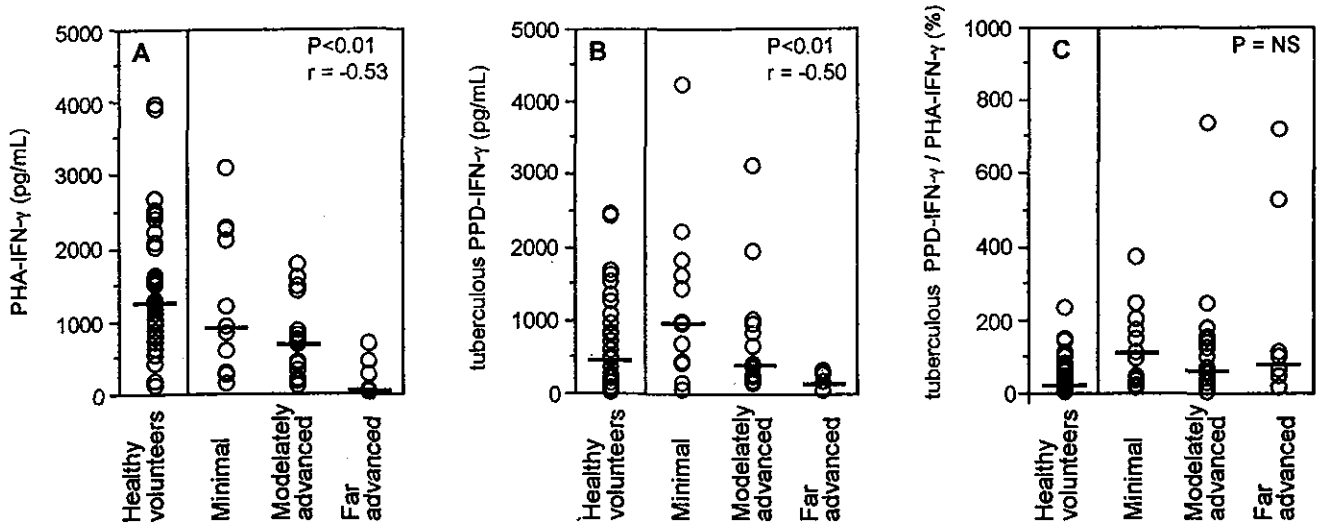


Fig. 4. Correlation between IFN-γ production and radiographic extent of pulmonary tuberculosis. The horizontal bars represent the median values. Disease extent is expressed as minimal, moderately advanced or far advanced disease. The correlation was evaluated by the Spearman method, excluding the results of healthy subjects. A: PHA-stimulated IFN-γ, B: *M. tuberculosis* PPD-stimulated IFN-γ, C: *M. tuberculosis* PPD-stimulated IFN-γ as a percentage of PHA-stimulated IFN-γ.

tuberculous PPD-IFN-γ was reduced in patients with tuberculosis (Sanchez et al., 1994; Zhang et al., 1995; Hirsch et al., 1999). Recent studies reported that a high response to tuberculous PPD-IFN-γ was observed in patients with minimal tuberculosis, whereas low responses were observed in those with advanced disease (Sodhi et al., 1997; Ellner et al., 2000). In our study, tuberculous PPD-IFN-γ was also suppressed with the disease extent, and the study population comprised more patients with minimal disease. The discrepancy between our results and those of previous studies may be due to differences in the study populations. Tuberculous PPD-IFN-γ was increased by tuberculosis infection and decreased with the disease extent. Taken together, these findings indicate that the interpretation of tuberculous PPD-IFN-γ is not always conclusive. Since tuberculous PPD-IFN-γ/PHA-IFN-γ is normalized by the baseline immune response to PHA, this percentage may be preferable for the diagnosis of tuberculosis.

Advanced pulmonary lesions correlated with depressed lymphocyte and $CD4^+$ cell counts, and with a reduced amount of PHA-IFN-γ. In contrast, tuberculous PPD-IFN-γ/PHA-IFN-γ did not correlate with disease extent. Previous studies also reported that the severity of tuberculosis is associated with depression of lymphocyte and $CD4^+$ cell counts in HIV-negative patients (Jones et al., 1997). Furthermore, IFN-γ production in response to tuberculous PPD or PHA is deficient in some patients with multidrug-resistant tuberculosis (McDyer et al., 1997). IFN-γ inhalation provides some clinical benefit in the treatment of multi-drug resistant tuberculosis (Condos et al., 1997). Thus, lymphocytes in advanced pulmonary tuberculosis may have quantitative and qualitative defects, and further studies are needed to clarify whether these defects are involved in treatment failure of tuberculosis.

The present study included some limitations. First, healthy volunteers were not matched to patients by age and sex. In order to reduce this bias, the results were statistically adjusted by using the multivariate analysis. Second, IFN-γ levels of patients and healthy volunteers overlapped, although the statistical differences were observed. Macrophages and $CD4^+$ cells with Th1 phenotype play an important role in protective immunity against *Mycobacterium*, and these cells secrete interleukin-12 (IL-12), IL-18, and tumor necrosis factor (TNF)-α as well as IFN-γ (Schluger and Rom, 1998). The roles of these various factors should be further investigated to evaluate the precise immune response in each patient.

In conclusion, while tuberculous PPD-IFN-γ/PHA-IFN-γ may be useful for the diagnosis of pulmonary tuberculosis; this parameter did not correlate with the disease severity. Instead, whole blood PHA-IFN-γ could be considered as a marker of disease severity.

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# Effects of DQ-113, a New Quinolone, against Methicillin- and Vancomycin-Resistant *Staphylococcus aureus*-Caused Hematogenous Pulmonary Infections in Mice

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**We compared the effects of DQ-113, a new quinolone, to those of vancomycin (VCM) and teicoplanin (TEIC) in murine models of hematogenous pulmonary infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and VCM-insensitive *S. aureus* (VISA). The MICs of DQ-113, VCM, and TEIC for MRSA were 0.125, 1.0, and 0.5 μg/ml, respectively; and those for VISA were 0.25, 8.0, and 8.0 μg/ml, respectively. Treatment with DQ-113 resulted in a significant decrease in the number of viable bacteria in the lungs of the mice used in the MRSA infection model (counts in mice treated with DQ-113, VCM, and TEIC and control mice, 6.33 ± 0.22, 7.99 ± 0.14, 7.36 ± 0.20, and 8.47 ± 0.22 $\log_{10}$ CFU/lung [mean ± standard error of the mean], respectively [$P < 0.01$ for the group treated with DQ-113 compared with the group treated with VCM or TEIC or the untreated group]). Mice infected with VISA were pretreated with cyclophosphamide, and the survival rate was recorded daily for 10 days. At the end of this period, 90% of the DQ-113-treated mice were still alive, whereas only 45 to 55% of the mice in the other three groups were still alive ($P < 0.05$ for the group treated with DQ-113 compared with the group treated with VCM or TEIC or the untreated group]). DQ-113 also significantly ($P < 0.05$) reduced the number of viable bacteria in the lungs compared with those in the lungs of the other three groups (counts in mice treated with DQ-113, VCM, and TEIC and control mice, 5.76 ± 0.39, 7.33 ± 0.07, 6.90 ± 0.21, and 7.44 ± 0.17 $\log_{10}$ CFU/lung, respectively). Histopathological examination revealed milder inflammatory changes in DQ-113-treated mice than in the mice in the other groups. Of the antibiotics analyzed, the parameters of area under the concentration-time from 0 to 6 h ($AUC_{0-6}$)/MIC and the time that the $AUC_{0-6}$ exceeded the MIC were the highest for DQ-113. Our results suggest that DQ-113 is potent and effective for the treatment of hematogenous pulmonary infections caused by MRSA and VISA strains.**

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first identified in the 1960s and was reported to colonize the upper respiratory tract and to cause severe infections, such as pneumonia, pulmonary abscesses, and septicemia. MRSA infection develops mainly in inpatients with risk factors related to health care (5), although it has also recently been described in the general population (1). Glycopeptides, such as vancomycin (VCM) and teicoplanin (TEIC), are the most reliable therapeutic agents against infections caused by MRSA. However, the first report of a Japanese patient harboring an MRSA strain resistant to VCM appeared in 1996 (3). Subsequent isolation of several VCM-resistant *S. aureus* (or VCM-insensitive *S. aureus* [VISA]) strains from the United States, France, Korea, South Africa, and Brazil confirmed that the emergence of VCM resistance in *S. aureus* is a global issue (9). Thus, some new agents with activities against MRSA and VISA, such as linezolid, daptomycin, and quinupristin-dalfopristin, have been developed (6). Tanaka et al. (10) reported that DQ-113, a new quinolone-type antibacterial agent, showed potent in vitro activities against various bacteria, including multiple-resistant strains, such as MRSA, VISA, and penicillin-resistant *Streptococcus pneumoniae*. It is expected that DQ-113 will be effective against severe staphylococcal infections, such as pneumonia, septicemia, and pulmonary abscesses.

A murine model of pulmonary infection with *S. aureus* by intravenous injection of bacteria enmeshed in agar beads was previously established (8) to evaluate the efficacies of antibiotics and the pathogenesis of blood-borne staphylococcal pneumonia. In the present study, we used the model to evaluate the antibacterial and histopathological effects of DQ-113 against MRSA and VISA by comparing these effects with those of VCM and TEIC.

## MATERIALS AND METHODS

**Laboratory animals.** Six-week-old male specific-pathogen-free ddY mice (body weight, 25 to 30 g) were purchased from Shizuoka Agricultural Cooperative Association Laboratory Animals (Shizuoka, Japan). All animals were housed in a pathogen-free environment and received sterile food and water ad libitum in the Laboratory Animal Centre for Biomedical Science at Nagasaki University. The Ethics Review Committee for Animal Experimentation at our institution approved in advance all experimental protocols described in this study.

**Bacterial strain.** Two strains of *S. aureus* were examined. Strain NUMR101 was isolated clinically at Nagasaki University Hospital from blood samples of infected patients. Mu50, a VCM-insensitive strain, was kindly provided by K.

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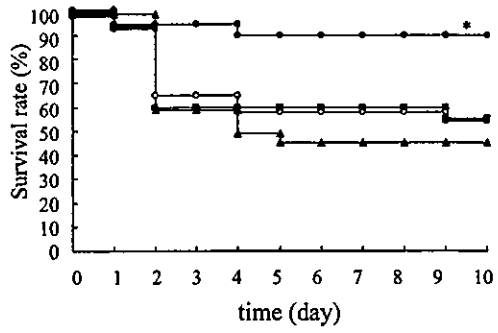


FIG. 1. Effects of DQ-113, VCM, and TEIC on survival rates of animals with hematogenous pulmonary infections caused by VISA. The mice were treated with one of the antibiotics after infection with VISA or were left untreated (controls). The survival rate was determined daily for 10 days. At 10 days, 90% of the mice in the DQ-113-treated group and 45 to 55% of mice in the other three groups (VCM- and TEIC-treated mice and untreated mice) were still alive. DQ-113 significantly improved the survival rate (*, $P < 0.05$). •, DQ-113; ▲, VCM; ▪, TEIC; □, control.

Hiramatsu (Juntendo University, Tokyo, Japan) (3). The bacteria were stored at −70°C in brain heart infusion broth (BBL Microbiology System, Cockeysville, Md.) supplemented with 10% (vol/vol) glycerol and 5% (wt/vol) skim milk (Yukijirushi Co., Tokyo, Japan) until use.

**MIC determinations.** DQ-113 (Daiichi Pharmaceutical Co., Tokyo, Japan) was dissolved in 0.01 N NaOH–2% glucose–1 mM phosphate-buffered saline. VCM (Shionogi Pharmaceutical Co., Tokyo, Japan) and TEIC (Aventis Pharmaceutical Co., Tokyo, Japan) were dissolved in sterile water immediately before use. The MIC of each agent was determined by the microplate dilution technique with Mueller-Hinton medium and an inoculum of $5 \times 10^5$ CFU/ml. The MIC was defined as the lowest concentration of the test drug that inhibited visible growth of the bacteria after 18 h of incubation at 37°C.

**Inoculum.** The method of inoculation was described previously (8). *S. aureus* was cultured on a Trypticase soy agar (BBL Microbiology Systems)-based sheep blood agar plate for 24 h at 37°C. The bacteria were suspended in endotoxin-free sterile saline and harvested by centrifugation (3,000 × *g*, 4°C, 10 min). The microorganisms were resuspended in cold sterile saline and diluted to $2 \times 10^9$ to $4 \times 10^9$ CFU/ml, as estimated by turbidimetry. The suspension was warmed to 45°C, and then 10 ml of the suspension was mixed with 10 ml of 4% (wt/vol) molten Noble agar (Difco Laboratories, Detroit, Mich.) at 45°C. The agar-bacterium suspension (1.0 ml) was placed in a 1.0-ml syringe, and the suspension was rapidly injected with a 26-gauge needle into 49 ml of rapidly stirred ice-cooled sterile saline. This resulted in solidification of the agar droplets into beads of approximately 200 μm in diameter. The final concentration of agar was 0.04% (wt/vol), and the final number of bacteria was $2 \times 10^7$ to $4 \times 10^7$ CFU/ml.

Because of the low level of pathogenicity of Mu50, mice infected with VISA were pretreated with cyclophosphamide (100 mg/kg) at 1 and 3 days before inoculation, and the survival rate was recorded daily over a period of 10 days (12).

**Experimental model.** We injected 0.20 to 0.25 ml of the agar beads that contained the bacteria and that were suspended in saline into the tail vein of each mouse (10 ml/g of body weight). Before the bacteria were enmeshed in the agar beads, we verified their numbers by inoculating duplicates of serial dilutions onto blood agar plates and counting the number of CFU after 48 h of incubation at 37°C. The method used to induce infection has previously been described in detail (8). Treatment commenced a day after inoculation by intraperitoneal administration of antibiotics. For the study with MRSA, 45 animals received one of the following eight treatments: DQ-113 (40 mg/kg of body weight/day; $n = 6$), VCM (40, 80, or 160 mg/kg/day; $n = 6$ each), TEIC (40, 80, or 160 mg/kg/day; $n = 6$, 5, and 5, respectively), or no treatment (controls; $n = 5$). Each drug was administered twice daily (every 12 h) for 7 days. We also conducted a survival study and a bacteriological study with VISA. Mice infected with VISA were pretreated with cyclophosphamide. In the survival study, the mice were treated every 12 h with 40 mg/kg/day for 10 days ($n = 20$). In the bacteriological study, 24 animals received one of the following four treatments: DQ-113 (40 mg/kg/day; $n = 6$), VCM (40 mg/kg/day; $n = 6$), TEIC (40 mg/kg/day; $n = 6$), or no treatment (controls; $n = 6$). We investigated the number of viable bacteria in the lungs after 7 days of treatment.

**Bacteriological, survival, and histopathological examinations.** Each group of animals was killed by cervical dislocation at specific time intervals. After exsanguination, the lungs were dissected and removed under aseptic conditions. The organs used for bacteriological analyses were homogenized and cultured quantitatively by serial dilution on blood agar plates. Lung tissue for histological examination was fixed in 10% buffered formalin and stained with hematoxylin-eosin.

**Lung and serum drug concentrations in mice.** The animals were killed by cervical dislocation at 0.25, 0.5, 1, 2, 4, and 6 h after treatment. Serum was separated after the blood had clotted. Four animals were used for each group. The lungs were removed, washed briefly, and cryohomogenized with saline. These samples were immediately frozen and stored at −80°C for a few days until the assay was performed. The concentration of DQ-113 was measured by the paper disk (bioassay) method (4). The test organism was *Bacillus subtilis* ATCC 6633. The concentrations of VCM and TEIC were measured by fluorescence polarization immunoassay (7). Pharmacokinetic parameters were calculated from the arithmetic mean concentrations in serum and lung tissue.

**Statistical analysis.** Bacteriological data were expressed as means ± standard errors of the means (SEMs). Survival data were compared by plotting Kaplan-Meier curves. Differences between groups were examined for statistical significance by the unpaired *t* test for MRSA and the log-rank test for VISA. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

## RESULTS

**Therapeutic effects of antibiotics.** Treatment with VCM (40 or 80 mg/kg/day) did not reduce the number of viable bacteria in the lungs relative to the number in the lungs of the controls (VCM at 40 mg/kg/day, 7.99 ± 0.14 $\log_{10}$ CFU/lung; VCM at 80 mg/kg/day, 8.15 ± 0.18 $\log_{10}$ CFU/lung; controls, 8.47 ± 0.22 $\log_{10}$ CFU/lung [$n = 6$, 6, and 5, respectively]). Treatment with VCM (160 mg/kg/day) or TEIC (40, 80, or 160 mg/kg/day) reduced the number of viable bacteria in the lungs relative to the number in the lungs of the controls (VCM at 160 mg/kg/day, 7.15 ± 018 $\log_{10}$ CFU/lung; TEIC at 40 mg/kg/day, 7.36 ± 0.20 $\log_{10}$ CFU/lung; TEIC at 80 mg/kg/day, 7.66 ± 0.18 $\log_{10}$ CFU/lung; TEIC at 160 mg/kg/day, 6.95 ± 0.27 $\log_{10}$ CFU/lung [$n = 6$, 6, 5, and 5, respectively]). In contrast, administration of DQ-113 at 40 mg/kg/day resulted in a significant decrease in the number of viable bacteria compared with the number in the other groups (6.33 ± 0.22 $\log_{10}$ CFU/lung [$n = 6$] [$P < 0.05$ versus the counts for the other groups]). The data are representative of those from three independent experiments. In the VISA study, 90% of mice treated with DQ-113 were still alive at the end of the study, while the survival rates were only 45 to 55% for the other three groups (Fig. 1). The differences in survival rates between the group treated with DQ-113 and the other three groups were significant ($P < 0.05$ for each comparison). The data are representative of those from two independent experiments. DQ-113 also significantly ($P < 0.05$) reduced the number of viable bacteria in the lungs compared with the number in the lungs of the other three groups (for DQ-113, VCM, TEIC, and the controls, 5.76 ± 0.39, 7.33 ± 0.07, 6.90 ± 0.21, and 7.44 ± 0.17 $\log_{10}$ CFU/lung, respectively [$n = 6$ for each group]).

**Histopathological examination.** At 7 days after treatment, microscopic examination of lung tissue specimens of mice infected with VISA Mu50 showed lung abscesses consisting of a central zone comprising a bacterial colony with infiltration of acute inflammatory cells (Fig. 2). Findings for the mice treated with VCM (Fig. 2c) and TEIC (Fig. 2b) were similar to those for the control mice (Fig. 2a). DQ-113-treated mice (Fig. 2d)

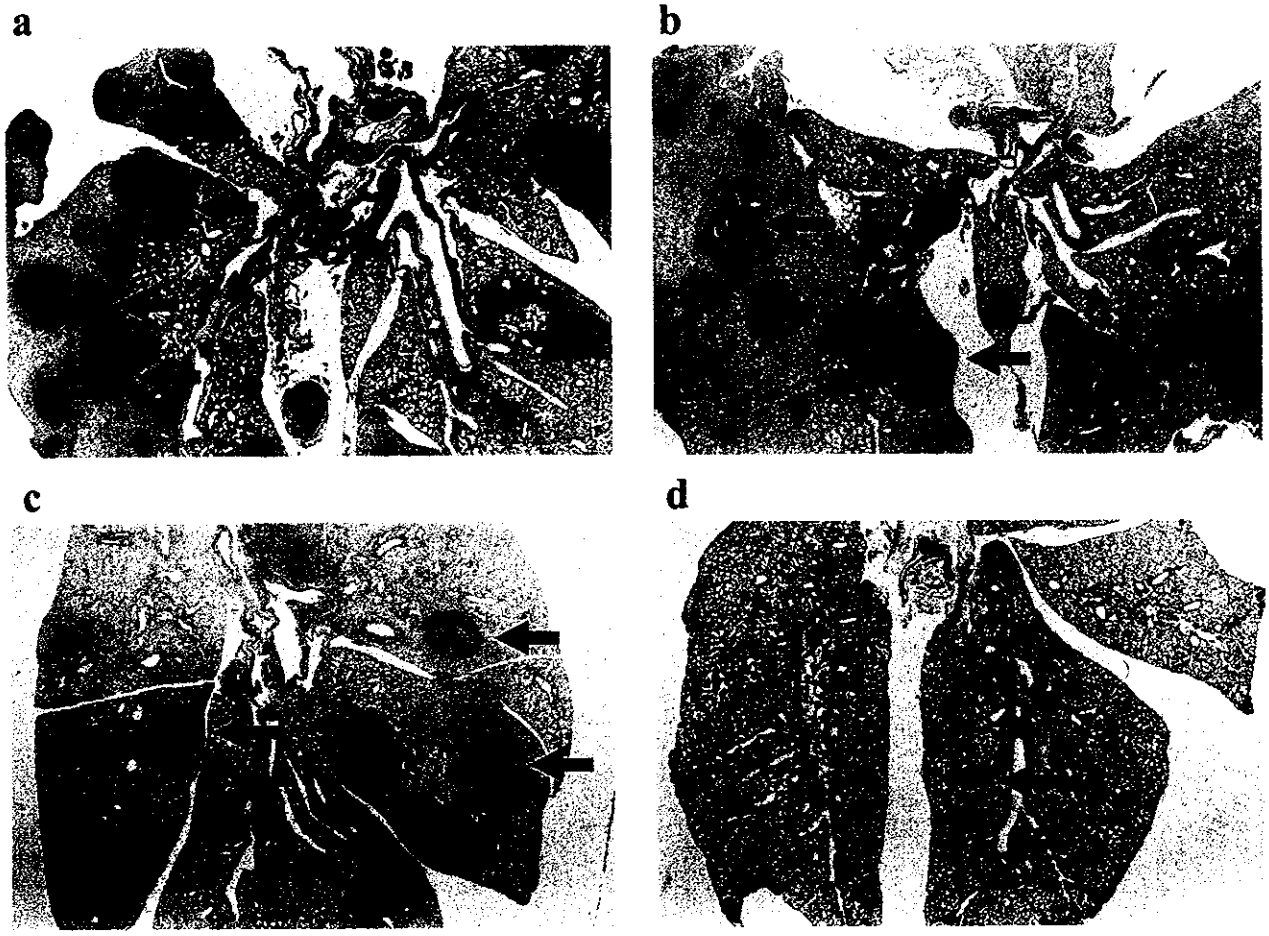


FIG. 2. Histopathological examination of lung specimens from mice killed 10 days after treatment. Each specimen exhibited typical features of lung abscesses consisting of a central zone comprising a bacterial colony with infiltration of acute inflammatory cells (hematoxylin and eosin stain). Arrows show the lung abscesses. (a) control; (b) TEIC-treated group; (c) VCM-treated group; (d) DQ-113-treated group. Note that the severity of the inflammatory process is less in DQ-113-treated mice than in the mice in the other groups. Magnifications, ×5.

exhibited fewer abscesses and milder inflammatory processes relative to those for the other groups.

**Serum and lung DQ-113, VCM, and TEIC concentrations in mice.** Figure 3a and b shows the mean concentrations of DQ-113, VCM, and TEIC in the sera and lungs of the mice 0.25, 0.5, 1, 2, 4, and 6 h after administration. These data are for MRSA-infected mice, and each drug was administered at 20 mg/kg once a day after inoculation. The peak concentrations of DQ-113, VCM, and TEIC in serum were 1.30 ± 0.23, 20.46 ± 2.98, and 60.40 ± 1.47 μg/ml, respectively (mean ± SEM; $n$ = 4). The peak concentrations of DQ-113, VCM and TEIC in lung tissue were 3.38 ± 0.19, 15.51 ± 4.04, and 3.18 ± 1.34 μg/ml, respectively (mean ± SEM; $n$ = 4). Table 1 shows the pharmacodynamic and pharmacokinetic parameters in the

TABLE 1. Selected pharmacokinetic parameter estimates for antibiotics in lung tissues in MRSA and VISA study[a]

| Antibiotic | Strain | MIC (μg/ml) | $AUC_{0-6}$ (μg · h/ml) | $C_{max}$ (μg/ml) | $t_{1/2}$ (h) | $AUC_{0-6}$/MIC (h) | $AUC_{0-6}$ > MIC (μg · h/ml) | $AUIC_{0-6}$ (h) |
|---|---|---|---|---|---|---|---|---|
| DQ-113 | MR101 | 0.125 | 5.52 | 3.38 | 1.62 | 44.14 | 5.52 | 44.14 |
| | Mu50 | 0.25 | | | | 22.07 | 5.31 | 21.25 |
| VCM | MR101 | 1 | 36.36 | 15.51 | 6.26 | 36.36 | 36.34 | 36.34 |
| | Mu50 | 8 | | | | 4.54 | 8.68 | 1.08 |
| TEIC | MR101 | 0.5 | 5.32 | 3.18 | 2.25 | 10.65 | 4.08 | 8.15 |
| | Mu50 | 8 | | | | 0.67 | 0 | 0 |

[a] The pharmacokinetic data are for MRSA-infected mice. Each drug was administered at 20 mg/kg once a day after inoculation. Pharmacokinetic parameters were calculated from the arithmetic means of the concentrations in lung tissue (mean values for four animals). $C_{max}$, maximum concentration; $t_{1/2}$, half-life.

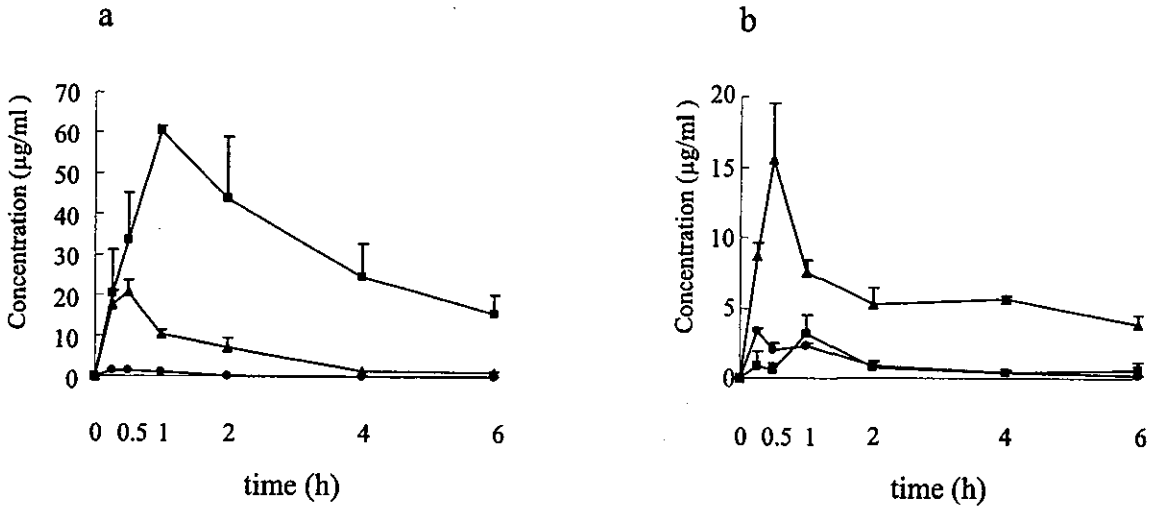


FIG. 3. Pharmacokinetics of DQ-113 (20 mg/kg), VCM (20 mg/kg), and TEIC (20 mg/kg) in the sera (a) and lungs (b) of MRSA-infected mice. Each drug was administered intraperitoneally after infection. The results are presented as means ± SEMs. ●, DQ-113; ▲, VCM; ■, TEIC.

lung tissues of mice with MRSA and VISA infections. The MICs of DQ-113, VCM, and TEIC for NUMR101 were 0.125, 1.0, and 0.5 μg/ml, respectively; and those for Mu50 were 0.25, 8.0, and 8.0 μg/ml, respectively. Of the antibiotics analyzed, the parameters of the area under the concentration-time curve from 0 to 6 h ($AUC_{0-6}$)/MIC and the time that the $AUC_{0-6}$ exceeded the MIC ($AUC_{0-6}$ > MIC)/MIC ($AUIC_{0-6}$) were the highest for DQ-113.

## DISCUSSION

In the present study, we were successful in inducing severe pneumonia and lung abscesses in VISA Mu50-infected immunocompromised mice, resulting in the death of 60% of the mice at 10 days after infection. While VCM and TEIC had no effect on the survival rate, DQ-113 protected the mice against fatal pneumonia and resulted in a significant reduction in the mortality rate. VCM and TEIC had insufficient effects in the model because they poorly penetrated lung tissue. VCM and TEIC were effective in a dose-dependent manner in the MRSA study.

In our model of hematogenous pulmonary infection, the new oxazolidinone antimicrobial linezolid significantly reduced the number of MRSA organisms and improved the survival rates of mice infected with VISA compared to the effects of VCM and TEIC (12). Our present data suggest that DQ-113 is a potent antimicrobial agent against VISA infection as well as MRSA, similar to linezolid.

VCM-resistant *S. aureus* was recently isolated in the United States. In a study published by the Centers for Disease Control and Prevention (2), the MICs of VCM, TEIC, and oxacillin for VISA were >128, 32, and 16 mg/ml, respectively. The isolate contained the *vanA* VCM resistance gene from enterococci, which is consistent with the glycopeptide MIC profiles (2).

The new oxazolidinone antimicrobial linezolid has been approved for use for the treatment of infections caused by various gram-positive bacteria, including MRSA and VCM-resistant enterococci. However, one MRSA strain resistant to linezolid has already been isolated from a patient treated with this agent for dialysis-associated peritonitis (11). These reports emphasize the need to develop antimicrobial agents potent against VISA. The available in vitro data (10) and the present results suggest that DQ-113 is a promising and potent candidate. In the pharmacokinetic study, the AUC/MIC and AUIC values for DQ-113 were the highest of those for the antibiotics analyzed. A recent brief report showed that DQ-113 accumulates at higher concentrations than other quinolones, suggesting that both its high intracellular concentrations and its inhibitory activities against target enzymes contribute to its potent antibacterial activity (M. Tanaka, T. Akasaka, Y. Onodera, M. Yoshihara, T. Takemura, and K. Sato, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 552, 2001).

In conclusion, we have demonstrated in the present study that DQ-113, a novel antibacterial quinolone, effectively reduced the number of bacteria in MRSA and VISA hematogenous infection models and significantly improved the rates of survival of immunocompromised mice infected with VISA compared with the rates achieved with VCM and TEIC.

## ACKNOWLEDGMENT

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# Analysis of Deaths During the Severe Acute Respiratory Syndrome (SARS) Epidemic in Singapore

## Challenges in Determining a SARS Diagnosis

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• ***Context.***—**An outbreak of severe acute respiratory syndrome (SARS), an infectious disease attributed to a novel coronavirus, occurred in Singapore during the first quarter of 2003 and led to 204 patients with diagnosed illnesses and 26 deaths by May 2, 2003. Twenty-one percent of these patients required admission to the medical intensive care unit. During this period, the Center for Forensic Medicine, Health Sciences Authority, Singapore, performed a total of 14 postmortem examinations for probable and suspected SARS. Of these, a total of 8 were later confirmed as SARS infections.**

***Objective.***—**Our series documents the difficulties encountered at autopsy during the initial phases of the SARS epidemic, when the pattern of infection and definitive diagnostic laboratory criteria were yet to be established.**

***Design.***—**Autopsies were performed by pathologists affiliated with the Center for Forensic Medicine, Health Sciences Authority, Singapore. Tissue was accessed and read at the Tan Tock Seng Hospital, Singapore, and at the Armed Forces Institute of Pathology, Washington, DC. Autopsy tissue was submitted to the Virology Department, Singapore General Hospital, for analysis, and in situ hybridization for the SARS coronavirus was carried out at the National Institute of Infectious Diseases, Tokyo, Japan.**

***Results.***—**Thirteen of 14 patients showed features of diffuse alveolar damage. In 8 patients, no precipitating etiology was identified, and in all of these patients, we now have laboratory confirmation of coronavirus infection. Two of the 8 patients presented at autopsy as sudden unexpected deaths, while the remaining 6 patients had been hospitalized with varying lengths of stay in the intensive care unit. In 3 patients, including the 2 sudden unexpected deaths, in situ hybridization showed the presence of virally infected cells within the lung. In 4 of the 8 SARS patients, pulmonary thromboemboli were also recognized on gross examination, while one patient had marantic cardiac valvular vegetations.**

***Conclusions.***—**It is unfortunate that the term *atypical pneumonia* has been used in conjunction with SARS. Although nonspecific by itself, the term does not accurately reflect the underlying dangers of viral pneumonia, which may progress rapidly to acute respiratory distress syndrome. We observed that the clinical spectrum of disease as seen in our autopsy series included sudden deaths. This is a worrisome finding that illustrates that viral diseases will have a spectrum of clinical presentations and that the diagnoses made for such patients must incorporate laboratory as well as clinical data.**

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Severe acute respiratory syndrome (SARS), an infectious disease attributed to a novel coronavirus,[1,2] was imported into Singapore in March 2003 by a patient who had traveled to Hong Kong[3] and who had stayed in the same hotel and same floor as the physician from Guangdong who is believed to be the source of infection for the outbreak in Canada.[4] This index case subsequently led to an outbreak of SARS in Singapore, with a total of 204 infections (103 of which were infected by 5 sources[5]) and 26 deaths by the beginning of May.[6]

Singapore had 4 clusters of infections during the 2-month period from mid March to the beginning of May.[3] Three were centered on hospitals (Tan Tock Seng Hospital with 1163 beds, Singapore General Hospital with 1502 beds, and National University Hospital with 943 beds[7]), while a fourth group developed around a vegetable wholesale center at Pasir Panjang, Singapore.

Approximately 75% of the SARS infections in Singapore have been traced to hospitals, and about 41% of the SARS

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**Table 1. Correlation Between Clinical Diagnosis and Main Pathology Findings***

| Case No. | Sex/ Age | Clinical Diagnosis | Right Lung, g | Left Lung, g | External Examination | Others |
|---|---|---|---|---|---|---|
| | | | **Confirmed SARS Cases** | | | |
| 1 | M/50 y | SARS (P), multiple organ failure | 1507 | 1497 | Multiple hemorrhagic infarcts, both lungs; subpleural hemorrhages | Organizing thrombi with occlusion of segmental pulmonary arteries, lungs; deep vein thrombosis; corticosteroid therapy, 9 d; mechanical ventilation, 4 d |
| 2 | M/39 y | SARS (P), acute myocardial infarct, septic shock | 1367 | 1194 | Multiple hemorrhagic infarcts, both lungs; multiple subpleural hemorrhages; red and gray hepatization | Organizing thrombi with occlusion of segmental pulmonary arteries, lungs; marantic vegetations: tricuspid, mitral, and aortic valves plus systemic infarcts; deep vein thrombosis; corticosteroid therapy, 9 d; mechanical ventilation, 8 d |
| 3 | F/29 y | SARS (P), septic shock, multiple organ failure | 1034 | 930 | Hemorrhagic and pale infarcts, both lobes; gray hepatization | Fragmented thrombi with occlusion of segmental pulmonary arteries, lungs; focal nodular hyperplasia, liver; deep vein thrombosis; thrombosis of paraovarian and parauterine veins; corticosteroid therapy, 3 d; mechanical ventilation, 6 d |
| 4 | M/68 y | Suspected SARS (S), acute myocardial infarct, hypertension, diabetes mellitus, end-stage renal failure on hemodialysis, ischemic heart disease, gout | 841 | 666 | Hypostatic congestion and consolidation; no infarct seen | Subendocardial infarct with coronary occlusive disease (75% occlusion); mechanical ventilation, <1 d |
| 5 | M/63 y | SARS (P), multiple organ failure, ischemic heart disease, congestive cardiac failure | 1254 | 1074 | Firm with multiple small cystic spaces, 5 mm each; no infarct | Coronary vessels: 10% occlusion; nephrosclerosis with infarct; mechanical ventilation, 2 d |
| 6 | M/38 y | Unable to exclude SARS (S), bilateral pneumonia, hypertension | 1316 | 1179 | Meaty, reddish appearance with scattered subpleural hemorrhages | Nonocclusive pulmonary artery thrombus, 4.5 cm; mechanical ventilation, <1 d |
| 7 | F/67 y | Unable to exclude SARS (S), fever, BID | 770 | 580 | Firm, patchy, red hepatization; severe congestion | |
| 8 | M/43 y | Unable to exclude SARS (S), ? dengue, ? upper respiratory tract illness | 750 | 650 | Diffuse consolidation; severely congested | |
| | | | **Non-SARS Cases** | | | |
| 9 | F/16 mo | Unable to exclude SARS (S), viral pneumonitis, status asthmaticus | 318 | 261 | Diffuse reddish appearance, whitish speckling; subpleural pale areas | Nil of note; mechanical ventilation, 2 d |
| 10 | M/87 y | Unable to exclude SARS (S), nosocomial pneumonia, sepsis, cardiac failure, ischemic heart disease | 1309 | 434 | Gray hepatization with nodular areas | Iliac artery aneurysm; hypertensive nephrosclerosis; mechanical ventilation, 4 d |
| 11 | M/47 y | Probable SARS (P), multiple organ failure, septic shock, DIVC | 1689 | 1158 | Multiple subpleural hemorrhagic subpleural infarcts with central softening; gray hepatization | Fibrin thrombi within glomeruli; mechanical ventilation, 2 d |
| 12 | M/70 y | Probable SARS (P), multiple organ failure, intra-abdominal abscess, hypertension, gout, acute renal failure, diabetes mellitus | 616 | 604 | Bronchopneumonia | Perforated duodenum; hypertensive and diabetic nephrosclerosis; mechanical ventilation, >7 d |
| 13 | M/88 y | Unable to exclude SARS (S), nosocomial pneumonia, ARDS, multiple fractures | 460 | 788 | Congestion with pneumonia and small 5-mm cavities | Multiple fractures; hypertensive nephrosclerosis; mechanical ventilation, 7 d |
| 14 | M/47 y | Unable to exclude SARS (S), fever, BID | 934 | 813 | Multiple pleural petechial hemorrhages | Nil of note |

* SARS indicates severe acute respiratory syndrome; BID, brought in dead; DIVC, disseminated intravascular coagulation; and ARDS, acute respiratory distress syndrome. SARS status is indicated as probable (P) or suspect (S).

patients were health care workers. In our experience, 21% (45) of the SARS patients required admission to the medical intensive care unit (ICU) and, of these, 84% required intubation for acute lung injury ($P_{AO_2}/F_{IO_2} \leq 300$) or acute respiratory distress syndrome (ARDS) ($P_{AO_2}/F_{IO_2} \leq 200$).[8]

During this period, the Center for Forensic Medicine, Health Sciences Authority, Singapore, performed 14 autopsies (Table 1) from different hospitals in Singapore, 6 of which were for probable SARS cases and 8 of which were for suspected SARS cases. Nine of these were mandated by the Director of Medical Services, Singapore, under the

**Table 2. Clinical Profile of Autopsy Cases***

| | SARS | Non-SARS |
|---|---|---|
| Sex: male-female ratio | 6:2 | 5:1 |
| Age: mean (range) | 50 y (29–68) | 56 y (1.5–88) |
| Length of hospital stay | 6.1 d (0–11) | 11.5 d (0–40) |
| Comorbid factors | Diabetes mellitus, end-stage renal failure, ischemic heart disease, hypertension | Diabetes mellitus, hypertension, multiple fractures, sepsis, empyema gall bladder, asthma, HIV |
| Symptoms | | |
| Fever, mean (range) | 37.6°C (35–38.9) | 39°C (37.8–40.5) |
| Cough | 4/8 patients | 3/6 patients |
| Shortness of breath | 4/8 | 2/6 |
| Myalgia | 3/8 | 0/6 |
| Diarrhea | 2/8 | 0/6 |
| Chest pain | 0 | 1/6 |
| Others | Headache, giddiness, runny nose | Uncontrolled diabetes, road traffic accident, abdominal pain |
| Chest x-ray film, initial changes† | Normal, 1/6 patients; patchy ground-glass opacities, 5/6 | Reticular linear shadows, 1/5; lobar consolidation, 1/5; normal, 2/5; fractured ribs, 1/5 |

| Hematology Component (Reference Range) | SARS, Mean (Range)‡ | | Non-SARS (Based on 3 Patients Admitted Immediately to the ICU), Mean (Range)§ |
|---|---|---|---|
| | Hospital | Medical ICU | |
| Total white blood cell count (4–10 × $10^3/\mu L$) | 7.5 (66–88.5) | 10.9 (5.4–14) | 8.5 (5–11.8) |
| Neutrophils (40%–70%) | 76.55 (66–88.5) | 85.4 (67–93) | 71.2 (47–86.6) |
| Lymphocytes (18%–43%) | 15 (10–24) | 10.6 (5.8–32.8) | 18.4 (4–44) |
| Platelets (160–390 × $10^3/\mu L$) | 173 (92–287) | 232 (70–430) | 108 (53–199) |
| LDH (180–380 U/L) (in 3 patients) | 845 (649–1125) | 1434 (748–2101) | 597 (only 1 patient, case 11) |

* SARS indicates severe acute respiratory syndrome; HIV, human immunodeficiency virus; ICU, intensive care unit; and LDH, lactate dehydrogenase.

† In 6 SARS patients and 5 non-SARS patients on admission; 2 SARS patients and 1 non-SARS patient were not admitted.

‡ In 6 SARS patients, on admission to the hospital and on admission to the ICU.

§ Not including case 9, the pediatric case, and case 12, the uncontrolled diabetic patient with multiple episodes in the ICU.

Infectious Diseases Act.[9] Three of the 14 cases presented as sudden unexpected deaths at the forensic service, 2 of which were later classified as deaths due to SARS.

## MATERIALS AND METHODS

All autopsies were carried out by pathologists (P.C., E.S.T., and K.B.T.) at the Health Sciences Authority, Singapore. Twelve were performed using positive air purifying respirators, and 2 were performed using routine precautions currently in place with N95 masks, disposable visors, gloves, protective gowns, and aprons.

Postmortem tissue was sent to the Tan Tock Seng Hospital, Singapore, and the Armed Forces Institute of Pathology, Washington, DC, for histopathology. Stains for macrophages and lymphocytes (CD68 and CD3, Dako Cytomation, Glostrup, Denmark) were also performed using the Ventana detection kit (Ventana Medical Systems, Tucson, Ariz).

Tissue was taken at autopsy for immunofluorescent antigen detection of respiratory viruses using the following methods: (1) polymerase chain reaction for coronavirus with a range of primers, including SAR1a/as and BNIoutS2/As2[2] and Cor1/2 (sense 5′-CAC CGT TTC TAC AGG TTA GCT AAC GA-3′ and antisense 5′-AAA TGT TTA CGC AGG TAA GCG TAA AA-3′) from the Government Virus Unit, Hong Kong,[10] and (2) viral isolation on a variety of cell lines, including Vero cells. Immunoglobulin M and G (IgM and IgG) antibodies to the coronavirus were detected using an immunofluorescent antibody assay. These assays were performed in the Virology Department, Singapore General Hospital (A.E.L., K.P.C., and L.L.E.O.).

In an attempt to localize the virus, unstained sections of the lung from the 8 cases with a positive polymerase chain reaction for the SARS coronavirus were submitted to the National Institute of Infectious Diseases, Tokyo, Japan, for detection of SARS using the recently described in situ hybridization-AT tailing technique with catalyzed signal amplification (T.S. and N.N.).[11]

Data from the Tan Tock Seng Hospital were collated by the Department of Diagnostic Radiology (G.J.L.K. and G.W.), the Medical ICU (D.Y.H.T.), and the Communicable Disease Center (Y.S.L.).

## RESULTS

Table 1 summarizes the clinical diagnoses and significant pathology findings with special reference to the pulmonary system in SARS and non-SARS cases. Table 2 gives the clinical profile of the SARS patients, and Table 3 details the virology and other microbiology findings in SARS and non-SARS cases. Table 4 gives details of the in situ hybridization-AT tailing–catalyzed signal amplification staining in the SARS cases.

### Clinical Findings

Eleven inpatients with progressive pulmonary symptoms subsequently underwent an autopsy for a clinical diagnosis of SARS or suspected SARS. Nine either had a history of exposure to SARS or had traveled to an affected area. Two (patients 9 and 11) were referred for autopsy because of a short history of fever, a rapid progression of disease, and radiologic findings that were suggestive of SARS. Three were routine coroner autopsies at which the forensic pathologist thought the pulmonary findings were suggestive of ARDS[12] but for which there was no apparent risk factor.

Eight patients (6 males and 2 females; mean age, 50 years) subsequently had laboratory confirmation of SARS infections, while 6 patients (5 males and 1 female; mean age, 56 years, including a pediatric patient, or 67 years, if the pediatric patient was excluded) were negative for coronavirus. Both groups had patients with comorbid conditions (Table 2).