Significantly higher cytokine and chemokine levels in patients with Japanese spotted fever compared with those with tsutsugamushi disease

Running title: Higher Cytokine Levels in Japanese Spotted Fever

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ABSTRACT

Tetracyclines are administered to cure Japanese spotted fever (JSF) and tsutsugamushi disease (TD). It is generally said that the clinical course of JSF is worse despite antibiotic treatment than that of TD. The precise mechanism underlying the more severe clinical course of JSF is not fully understood. We therefore examined whether a differential cytokine profile between these two infectious diseases contributes to the difference in clinical severity. The serum concentrations of various cytokines (TNF-α, IL-6 and IFN-γ) and chemokines (IL-8, IP-10, MCP-1, MIP-1α, MIP-1β and eotaxin) were measured in 32 TD and 21 JSF patients. Results showed that serum levels of TNF-α in the acute phases of TD and JSF were significantly increased with a higher concentration of TNF-α in patients with JSF (mean 39.9 pg/ml) compared with those with TD (mean 13.8 pg/ml). Comparatively higher levels of other cytokines and chemokines (IL-6, IFN-γ, IL-8, IP-10, MCP-1, MIP-1α and MIP-1β) were also observed in the acute phase of JSF. The clinical severity score (3.67 ± 1.71) of JSF patients was higher than that of TD patients (1.47 ± 0.77). Our findings revealed that the cytokine and chemokine levels in the acute phase of JSF were significantly higher than...
those in the acute phase of TD. The differential cytokine levels may be
related to the difference in clinical severity between JSF and TD.

Key word: Japanese spotted fever, tsutsugamushi disease, TNF-α, cytokine,
chemokine

INTRODUCTION

Scrub typhus, also known as tsutsugamushi disease (TD), is a mite-borne
infection caused by Orientia tsutsugamushi that occurs frequently in
Southeast Asia. The vector of this disease is the trombiculid mite, also
known as tsutsugamushi, which resides in mountainous areas and around
rivers throughout Southeast Asia. In Japan, the number of cases of TD is
approaching near 500 per year (1, 2). TD is characterized by fever, rash and
eschar (3). Both minocycline and doxycycline are effective for treating TD and
eradicating O. tsutsugamushi, and marked defervescence is observed in the
majority of patients within 24 h after commencement of tetracyclines (4, 5).

Japanese spotted fever (JSF) caused by Rickettsia japonica was first
reported in Japan in 1984 (6-9). Endemic areas of JSF are located along the
southwestern and central coastal areas of Japan with a warmer climate (9). Clinical manifestations (high fever, rash and tick bite eschar) of JSF are similar to those of TD (3, 10). Tetracyclines are recommended for clinically suspected cases of JSF (5), and complications such as pneumonia, meningitis, disseminated intravascular coagulation (DIC), and systemic inflammatory response syndrome (SIRS) leading to multiple organ failure may result if tetracyclines are not administered early (11, 12).

It is generally said that the clinical course of JSF is worse despite antibiotic treatment than that of TD. The mortality rate of TD was reported to be 7% in untreated classical cases, while the mortality rate of Rocky Mountain spotted fever in the pre-antibiotic era was 20%–25%, and the recent mortality rate, due to delay in the diagnosis and therapy, was reported to be 5% (13). Compared with these mortality rates, it is important to recognize that the mortality rate of JSF may be similar to that in patients with Rocky Mountain spotted fever, which is the most lethal rickettsiosis. The precise mechanism underlying the more severe clinical course of JSF is not fully understood, and in the future, a larger number of patients should be investigated. It has been hypothesized that fatal JSF is associated with
hypercytokinemia (14), however, no studies exist that elaborate cytokine levels in the acute phase of JSF.

In the present study, we measured the concentration of several cytokines and chemokines in patients with TD and JSF before and after the administration of minocycline, and compared these levels between the acute phases of JSF and TD. We believe our study is of great interest to physicians involved in the treatment of patients with rickettsial infection.

MATERIALS AND METHODS

Patient characteristics and diagnosis

This prospective study was approved by the Institutional Review Board of the Faculty of Medical Science of the University of Fukui. Serum cytokine and chemokine levels were examined in 53 Japanese patients (25 males and 28 females, age range 13–86 years) with confirmed rickettsial disease, which included 32 patients with confirmed *O. tsutsugamushi* infection diagnosed between 2003 and 2009 in Tanabe City, Wakayama Prefecture, Japan, and 21 patients with confirmed *R. japonica* infection diagnosed between 2007 and 2008 in the mountainous region of Misen, Shimane Prefecture, Japan.
Detailed history-taking and a physical examination for rash and eschar were performed in all patients (Table 1). The diagnosis of rickettsiosis was based on a rise in serum IgM antibody titer or a four-fold rise in serum IgG antibody titer to strains of *O. tsutsugamushi* or *R. japonica* using an indirect immunoperoxidase antibody test performed on paired serum samples collected during the acute (1–7 days after disease onset) and convalescent phases (14–21 days after onset). Its sensitivity was 99.1% and specificity was 98.9% from reactivity to IgM and IgG (15, 16).

**Modified clinical severity scoring of rickettsial infections**

We used the clinical severity scoring system which was presented by Kern et al. for Mediterranean spotted fever with minor modifications to adjust it to *tsutsugamushi* disease (17). Disease severity was evaluated using a modified scoring system based on the clinical scoring system reported by Iwasaki et al. (18). In brief, the score was calculated as the sum of different point values assigned to specific criteria: points were assigned to the clinical manifestations of fever (1 point or 2 point), severe myalgia (1 point), lymphadenopathy, hepatosplenomegaly (1 point), liver dysfunction (1 point),...
thrombocytopenia (1 point or 2 point), disseminated intravascular coagulation (DIC), and central nervous system disorder (3 point) (Table 2). These scores, with possible total points ranging from 0 to 15, were determined on the basis of laboratory data and major symptoms on admission.

Cytokines and chemokines in patients with TD and JSF

Sera taken from patients with TD and JSF were collected at the first clinic visit (acute phase) and 2 weeks after initiation of minocycline therapy (convalescent phase), and were frozen within 8 h of receipt and stored at −80 °C until analysis. In our experience, the freezing of sera does not affect the results on retesting (19). All serum cytokine and chemokine levels were simultaneously measured using multiplex bead immunoassays. Multiplex bead immunoassays (Bio-Plex Suspension Array System, BIO-RAD Laboratories, Inc., CA, USA) were used to quantify cytokines and chemokines simultaneously by following the manufacturer’s instructions (20). This novel immunoassay uses color-coded beads and permits the simultaneous detection of up to 100 cytokines and chemokines in a single
well of a 96-well microplate in just 3 h (21-23). The serum levels of the following cytokines and chemokines were assessed: tumor necrosis factor α (TNF-α), interleukin (IL)-6, interferon (IFN)-γ, IL-8, interferon inducible protein (IP)-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, and eotaxin. The concentration of each cytokine and chemokine in the acute and convalescent phases is reported.

Statistical analysis

All data are presented as mean ± standard deviation (SD). Student’s t-test was performed to analyze differences in the data using statistical software (Microsoft Excel 2010, Microsoft Corporation, Redmond, WA, USA). P < 0.05 was considered statistically significant.

RESULTS

Clinical features of TD and JSF

All clinically suspected cases of rickettsial infection underwent indirect immunoperoxidase antibody testing performed on paired serum samples
collected during the acute and convalescent phases. The IgM antibody titers were more than 1:640 during the acute phase in each case, and IgG antibody titers also increased more than 1:640 in the convalescent phase (data not shown). The following strains of *O. tsutsugamushi* were identified: Gilliam (n = 0), Karp (n = 2), Kato (n = 0), Irie/Kawasaki (n = 24), Hirano/Kuroki (n = 6), and Shimokoshi (n = 0). Patient demographics and clinical and laboratory data are summarized in Table 1. *O. tsutsugamushi* and *R. japonica* differ in antigenic determinant, and accordingly, there was no overlap of the serologic assays for TD and JSF. The peak temperatures of fever in patients with JSF were higher than those in patients with TD (39.2 ± 0.7 °C vs. 38.1 ± 0.7 °C) (Fig 1A). Moreover, patients with JSF had a higher clinical severity score (3.67 ± 1.71) than patients with TD (1.47 ± 0.77) (Fig 1B). Patients were given minocycline 100–200 mg/day intravenously or *per os* for 3–10 days, and all responded well to treatment.

**Serum cytokine and chemokine levels during minocycline treatment of TD and JSF**

The serum levels of TNF-α, IL-6, IFN-γ, IL-8, IP-10, MCP-1, MIP-1α and
MIP-1β were elevated in almost all patients during the acute phase of rickettsial disease (Fig 2 A-I). Compared with the acute phase, the serum concentrations of these cytokines and chemokines were significantly decreased in the convalescent phase. In contrast, eotaxin levels were unchanged in the acute and convalescent phases. We next examined whether serum levels of cytokines and chemokines were significantly different between the acute phases of TD and JSF. The mean concentrations of cytokines and chemokines in patients infected with O. tsutsugamushi or R. japonica are shown in Table 3A. Serum concentrations of TNF-α in patients with JSF (35.0 pg/ml) were significantly higher than those in patients with TD (13.8 pg/ml). Similarly, the serum levels of cytokines IL-6 (65.8 pg/ml vs. 11.6 pg/ml), IFN-γ (220.3 pg/ml vs. 73.4 pg/ml), and chemokines IL-8 (46.3 pg/ml vs. 13.2 pg/ml), IP-10 (12,298.3 pg/ml vs. 3,215.9 pg/ml), MCP-1 (1,365.8 pg/ml vs. 467.5 pg/ml), MIP-1α (53.9 pg/ml vs. 15.0 pg/ml) and MIP-1β (127.7 pg/ml vs. 88.5 pg/ml) in JSF were significantly higher than those in TD. However, there was no significant difference in the concentration of eotaxin between the acute phases of TD and JSF (67.1 pg/ml vs. 68.2 pg/ml). On the other hand, in convalescent phase, the serum levels of
TNF-α, IL-8 and MCP-1 in JSF were significantly higher than those in TD, and there was no significant difference in other cytokines and chemokines between TD and JSF (Table 3B).

A positive correlation exists between TNF-α and IL-8 (r = 0.876) (Fig 3A) and between TNF-α and IP-10 (r = 0.704) (Fig 3B) in the acute phase of JSF. In contrast, no significant correlation was observed between TNF-α and other cytokines and chemokines in the acute phase of TD.

DISCUSSION

In the present study, the levels of cytokines and chemokines in the acute phase of JSF were significantly higher than that of TD. Consistent with this, a positive correlation between TNF-α and IL-8 (r = 0.876) as well as TNF-α and IP-10 (r = 0.704) was observed in the acute phase of JSF. We speculate that the severity of JSF is related to hypercytokinemia. Systemic inflammatory reactions are regulated by a complicated cytokine network, and the production of proinflammatory cytokines and chemokines is essential in the immune response against pathogens. Regulation of cytokine and chemokine production is necessary for an appropriate host response:
however, tissue damage may result from activation of immune cells due to the overproduction of cytokines and chemokines. The unregulated and excessive systemic release of numerous cytokines and chemokines in a life-threatening infection can cause systemic disorders and lead to fatal consequences such as septic shock or SIRS (24, 25). We estimate that hypercytokinemia may contribute to the disease severity of rickettsiosis including JSF. For instance, fulminant TD has been associated with hemophagocytic syndrome (10), and this syndrome may represent a hyperreaction of the immune system mediated by an overactivated cytokine network during the advanced stages of rickettsial infection (19, 26-28). We previously reported that a downregulation of these cytokines and chemokines is observed after the administration of tetracyclines, and demonstrated a correlation between the severity of TD and the serum concentration of TNF-α in the acute phase of the disease, and showed that TNF-α levels are predictive of the severity of TD (18, 19, 29, 30). In relation to other spotted fever group, it is reported that Mediterranean spotted fever (MSF) is characterized by a Th1 cytokine profile, and the patient’s immune system responds by proinflammatory and immunoregulatory cytokine
production (IL-1β, TNF-α, IL-6, INF-γ, IL-8, IL-10, IL-12) that accompanies the rickettsial vasculitis and contributes to the healing process (31). It was not much different on cytokine profile between JSF and MSF. There were not the increases of some cytokines specific to JSF among the rickettsioses. It is generally said that the clinical course of JSF is worse despite antibiotic treatment than that of TD, and in cases of severe JSF with complications where monotherapy with either minocycline or doxycycline was not effective, such patients can be successfully treated using tetracyclines combined with quinolones (ciprofloxacin, levofloxacin, etc.) (32). However, in this study, although we examined some correlation between the severity of JSF and some cytokine level, we could not show a significant difference. It is unclear whether the severity of JSF is related to the serum concentration of cytokines and chemokines in the acute phase of the disease. Although the precise mechanism underlying the disease severity of JSF is not well understood, our study provides insight into the possible contribution of cytokines and chemokines in the pathogenesis of this disease.

In conclusion, the present study showed that the serum cytokine and chemokine levels were significantly higher in JSF than those in TD. We
suspect that the difference in cytokine and chemokine levels contribute to
the difference in disease severity between JSF and TD.

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excellent technical assistance and helpful discussions.

Conflict of interest: No conflict of interest to declare
FIGURE LEGENDS

Fig 1. (A) Comparison of the peak temperatures (B) and clinical severity scores of patients with tsutsugamushi disease (TD) and Japanese spotted fever (JSF).

Fig 2. Serum cytokine and chemokine levels during the acute and convalescent phases of TD and JSF. Serum cytokines TNF-α, IL-6 and IFN-γ (A-C) and chemokines IL-8, IP-10, MCP-1, MIP-1α, MIP-1β and eotaxin (D-I) were measured using Multiplex Bead Immunoassays. The concentrations of cytokines and chemokines in the acute and convalescent phases are shown.

Fig 3. (A) Relationship between TNF-α and IL-8 in the acute phase of JSF (r² = 0.876) (B) Relationship between TNF-α and IP-10 (r = 0.704).
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Fig 1

(A) **fever**

<table>
<thead>
<tr>
<th>°C</th>
<th>TD</th>
<th>JSF</th>
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<td>39.5</td>
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<tr>
<td>40</td>
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*(p < 0.001)*

(B) **Revised clinical severity score**

<table>
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</tr>
<tr>
<td>6</td>
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</table>

*(p < 0.001)*
Fig 2

(A)

log10(pg/ml)

acute phase
convalescent phase

TNF-α

P < 0.001

TD

100

log10(pg/ml)

acute phase
convalescent phase

TNF-α

P < 0.001

ISF
Fig 2 (B)

TD

JSF

IL-6

acute phase

convalescent phase

$P < 0.01$

$P < 0.001$

(pg/ml)

350

250

150

100

50

0

300

250

150

100

50

0

300

250

150

100

50

0

acute phase

convalescent phase

IL-6
Fig 2

**TD**

```
P < 0.001
```

**JSF**

```
P < 0.001
```

- **IFN-γ**
  - Acute phase
  - Convalescent phase

**levels (pg/ml)**

- 1000
- 900
- 800
- 700
- 600
- 500
- 400
- 300
- 200
- 100
- 0
Fig 2 (D)

TD

140 P<0.01 (pg/ml)

140 (pg/ml)

P<0.001

JSF

140 P<0.01 (pg/ml)

acute phase convalescent phase

IL-8

0 acute phase convalescent phase

IL-8
Fig 2

(E)

TD

IP-10

acute phase convalescent phase

P < 0.001

JSF

IP-10

acute phase convalescent phase

P < 0.001

pg/ml

35000

20000

15000

10000

5000

0
Fig 2

(F)

TD

(pg/ml)

3500

P < 0.01

JSF

(pg/ml)

3500

P < 0.001

 acute phase convalescent phase

MCP-1

 acute phase convalescent phase

MCP-1
Fig 2

(G)

TD

(P<0.05)

JSF

(P<0.001)

acute phase  convalescent phase
MIP-1α

acute phase  convalescent phase
MIP-1α
Fig 2

TD

(ng/ml)

H

300

P < 0.01

JSF

(ng/ml)

H

300

P < 0.001

acute phase
convalescent phase

MIP-1β

acute phase
convalescent phase

MIP-1β
### Table 1. Clinical characterization of TD and JSF

<table>
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<th>Variable</th>
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<tr>
<td></td>
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<td>%</td>
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<td>Eschar</td>
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<td>87.5</td>
</tr>
<tr>
<td>Skin rash</td>
<td>7</td>
<td>21.9</td>
</tr>
<tr>
<td>Fever (≥38°C)</td>
<td>21</td>
<td>65.6</td>
</tr>
<tr>
<td>Lymph node swelling</td>
<td>13</td>
<td>40.6</td>
</tr>
<tr>
<td>Thrombocytopenia (PLT&lt;50,000/mm³)</td>
<td>18</td>
<td>56.3</td>
</tr>
<tr>
<td>Liver dysfunction (ALT&gt;40 IU/L)</td>
<td>4</td>
<td>19.0</td>
</tr>
<tr>
<td>Disseminated intravascular coagulation (DIC)</td>
<td>1</td>
<td>3.1</td>
</tr>
<tr>
<td>Central nervous system disorder</td>
<td>1.47 ± 0.77</td>
<td>3.67 ± 1.71</td>
</tr>
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</table>

### Table 2. Revised clinical severity scoring system

1. Fever: >38°C  
   - >39°C: 2
2. Severe myalgia: 1
3. Lymph node swelling: 1
4. Hepatosplenomegaly: 1
5. Liver dysfunction (ALT>40 IU/L): 1
6. Thrombocytopenia with PLT counts: <100×10⁹/L: 1  
   - ≤50×10⁹/L: 2
7. Disseminated intravascular coagulation (DIC): 2
8. Central nervous system disorder: 3
Table 3: The difference in the cytokine and chemokine levels between TD and JSF

(A) Acute phase

<table>
<thead>
<tr>
<th></th>
<th>TD 32 patients (pg/ml ± standard deviation)</th>
<th>JSF 21 patients (pg/ml ± standard deviation)</th>
<th>t-test</th>
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<tr>
<td>TNF-α</td>
<td>15.8 ± 15.5</td>
<td>35.0 ± 27.5</td>
<td>P &lt; 0.001</td>
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<tr>
<td>IL-6</td>
<td>11.6 ± 17.2</td>
<td>65.8 ± 73.4</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>73.4 ± 78.1</td>
<td>220.5 ± 228.0</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>IL-8</td>
<td>13.2 ± 10.5</td>
<td>48.3 ± 88.3</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>IP-10</td>
<td>3,115.9 ± 2,786.1</td>
<td>12,768.8 ± 9,339.4</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>MCP-1</td>
<td>467.3 ± 492.0</td>
<td>1,365.8 ± 816.7</td>
<td>P &lt; 0.01</td>
</tr>
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<td>MIP-1α</td>
<td>35.0 ± 25.3</td>
<td>93.9 ± 41.0</td>
<td>P &lt; 0.01</td>
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<tr>
<td>MIP-1B</td>
<td>88.5 ± 62.5</td>
<td>127.7 ± 56.0</td>
<td>P &lt; 0.05</td>
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<tr>
<td>eotaxin</td>
<td>67.1 ± 31.7</td>
<td>60.2 ± 40.1</td>
<td>n.s.</td>
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(B) Convalescent phase

<table>
<thead>
<tr>
<th></th>
<th>TD 32 patients (pg/ml ± standard deviation)</th>
<th>JSF 21 patients (pg/ml ± standard deviation)</th>
<th>t-test</th>
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<td>TNF-α</td>
<td>4.1 ± 4.4</td>
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<td>880.8 ± 534.2</td>
<td>780.9 ± 808.2</td>
<td>n.s.</td>
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<td>MCP-1</td>
<td>28.9 ± 229.1</td>
<td>592.3 ± 592.3</td>
<td>P &lt; 0.001</td>
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<tr>
<td>MIP-1α</td>
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<td>9.5 ± 26.9</td>
<td>n.s.</td>
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<td>MIP-1B</td>
<td>63.7 ± 46.2</td>
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<td>eotaxin</td>
<td>77.7 ± 31.6</td>
<td>65.3 ± 40.4</td>
<td>n.s.</td>
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