

Persistence of *Orientia tsutsugamushi* in Humans

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We investigated the persistence of viable *Orientia tsutsugamushi* in patients who had recovered from scrub typhus. Blood specimens were available from six patients with scrub typhus who were at 1 to 18 months after the onset of the illness. The EDTA-treated blood specimens were inoculated into ECV304 cells, and cultures were maintained for 7 months. Sequencing of the 56-kDa type-specific antigen gene of *O. tsutsugamushi* was performed to ascertain the homology of isolates. *O. tsutsugamushi* was isolated from all six patients, and nucleotide sequences of isolates serially collected from each patient were identical in all five patients in whom nucleotide sequences were compared. One patient relapsed 2 days after completion of antibiotic therapy; two patients complained of weakness for 1 to 2.5 months after the illness; one patient underwent coronary angioplasty 6 months later; and one patient suffered from a transient ischemic attack 8 months later. This finding suggests that *O. tsutsugamushi* causes chronic latent infection, which may be associated with certain clinical illnesses, preceded by scrub typhus. Antibiotic therapy abates the symptoms of scrub typhus, but does not eradicate *O. tsutsugamushi* from the human body.

Key Words: *Orientia tsutsugamushi*; Scrub typhus; Persistence; Recurrence; Atherosclerosis

INTRODUCTION

Scrub typhus, also known as tsutsugamushi disease, is caused by *Orientia tsutsugamushi*, which is considered to be an acute infectious disease and to be successfully cured with antibiotics. Studies using the polymerase chain reaction (PCR) reveal that *O. tsutsugamushi* DNA in blood disappears gradually over 1 month after antibiotic treatment (1-4). It has not been clarified whether the DNA detected by PCR after the disappearance of symptoms of scrub typhus represents dead or viable bacteria, and its relevance and clinical significance have not been investigated. Although isolation of *O. tsutsugamushi* beyond 1 month after scrub typhus in humans has been reported sometimes (5-7), these cases are thought to be exceptional. Contrast to the short duration of scrub typhus in humans, persistence of *O. tsutsugamushi* is a common finding in mice (8-14). Ability of *O. tsutsugamushi* to persist in cell culture assay is also documented (15). Chloramphenicol and tetracyclines show bacteriostatic effects against *O. tsutsugamushi* in mice (10, 16); therefore, theoretically antibiotics cannot eradicate the bacterium from the human body. Although the immune system is known to be involved in the recovery of an individual from scrub typhus and in protection from rechallenge of *O. tsutsugamushi* (17), it is never been proven that this system can completely eradicate *O. tsutsugamushi* from the human body. In the present study, we investigated the persistence of viable *O. tsutsugamushi* and its associa-

tion with any clinical or laboratory abnormalities in patients who had recovered from scrub typhus.

MATERIALS AND METHODS

Patients

Blood specimens beyond 30 days after the onset of fever (defined as 'chronic phase') were available from 6 patients. Five patients (patient No. 1 to 5) were followed from the onset of scrub typhus diagnosed at our hospital between 2008 and 2009, and 1 patient (patient No. 6) was during follow up of other chronic illnesses after scrub typhus. Scrub typhus was diagnosed by the presence of fever and positive serology or blood nested PCR that was performed as described previously (3). Clinical and laboratory characteristics of the patients are summarized in Table 1.

In vitro culture and immunofluorescent staining

Details of cell culture and immunofluorescent (IF) staining were described previously (15). Briefly, 0.3 mL of EDTA-treated whole blood was inoculated into a monolayer of ECV304 cells (18) grown in a tissue culture flask (25 cm²). Twenty-four hours later, the inoculated blood was washed out with phosphate buffered saline, and culture was maintained in M199 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL) at 37°C in a humidified atmosphere containing 6% CO₂. Media were changed every

3 to 4 days, and cultures were maintained for 7 months without subculture. During the maintenance of the cell culture, cell cultures were inspected daily with optical microscopy (Olympus, Tokyo, Japan). After cytopathic changes were identified, a small amount of the ECV304 monolayer was aspirated with a pipette, and IF staining of the aspirate was performed with mouse polyclonal hyperimmune serum against the Boryong strain of *O. tsutsugamushi*. For negative control, blood from 5 healthy persons and 5 patients with unrelated illnesses (cerebrovascular accident [n = 2]; acute myocardial infarction [n = 2]; and diabetes mellitus [n = 1]) were processed as the same manner.

Polymerase chain reaction and sequencing of 56-kDa gene

To identify the genotype of cultured isolates and to compare nucleotide sequences of the isolates, the 56-kDa type-specific antigen (TSA56) gene of *O. tsutsugamushi* was amplified using PCR and then sequenced. Genomic DNA from the infected ECV304 cells was prepared with QIAamp DNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. PCR in detail was described previously (19). The primer set used for the

amplification of the first half TSA56 gene of *O. tsutsugamushi* were primer 1 (forward) (5'-TTTCGAACGTGTCTTTAAGC-3'; corresponding to nucleotide position -266 to -285 from the start codon of the 56-kDa gene based on the Gilliam strain) and primer 2 (reverse) (5'-ACAGATGCACTATTAGGCAA-3'; 847-865). Primer 3 (forward) (5'-ATGCTAATAAACCTAGCGCT-3'; 731-749) and primer 4 (reverse) (5'-CTAGAAGTTATAGCGTACACCTGCACTTGC-3'; 1546-1575) were used for amplification of the second half one. Primer 9 (5'-GTTTAGAATGGTTACCAC-3'; -36 to -53) and primer 7 (5'-AGCGCTAGGTTTATTAGCAT-3'; 731-749) were used for sequencing of the amplified first half of the TSA56 gene, and primer 8 (5'-TCCACATACACACCTTACGC-3'; 1459-1478) and primer 10 (5'-CCTAGCGTTACTCTGTCAAAG-3'; 742-763) were used for the amplified second half of the TSA56 gene. The first half of the TSA56 gene was amplified in a 50- μ L reaction mixture consisting of 5 μ L of template DNA, 250 μ M (each) deoxynucleotide triphosphate, 200 μ M each of primers 1 and 2, 1.25 U of *Taq* polymerase and 5 μ L of 10 \times PCR buffer (Takara Shuzo Co., Kyoto, Japan) in a Perkin-Elmer model 9600 thermocycler (Perkin-Elmer Cetus Instruments, Norwalk,

Table 1. Characteristics, clinical features, and laboratory results of the patients

Patient No.	Sex/age (yr)	Underlying illness	Antibiotics, duration of treatment	Duration after the onset of scrub typhus	Clinical feature, laboratory finding, and genotype of <i>O. tsutsugamushi</i> when blood specimen was obtained							
					Fever/eschar/rash	WBC (μ L)	CRP (mg/L)	ESR (mm/h)	AST/ALT (IU/L)	Anti- <i>Orientia</i> antibody (reciprocal titer)	Blood nested PCR	Cell culture/genotype of the isolate (GenBank accession number)/day to identify the growth of <i>O. tsutsugamushi</i> firstly
1	F/59	Hypertension	Azithromycin, 7 d; doxycycline, 7 d	7 d	+/+/+; shock, pneumonia	5,100	310	8	230/149	1,280	Positive	Positive/Yonchon (HQ660209)/63 d
				1 m	Weakness, anorexia	4,290	1.0	36	31/102	2,560	Positive	Positive/Yonchon (HQ660210)/83 d
2	F/48	None	Azithromycin, 3 d; Doxycycline, 10 d	13 d	+/+/+	ND	ND	ND	ND	ND	ND	ND
				2.5 m	Weakness	3,800	0.5	11	28/16	320	Positive	Positive/Boryong (HQ660214)/53 d
3	F/69	Diabetes	Azithromycin, 3 d	17 d	+/-/-	5,400	ND	ND	72/53	160	Positive	Positive/Boryong (HQ660198)/51 d
				27 d	None	6,400	ND	ND	22/25	320	Positive	Positive/Boryong (HQ660199)/ 63 d
				5 m	None	5,600	0.3	14	24/15	160	Negative	Positive/Boryong (HQ660200)/ 70 d
				8 m	None	5,700	0.3	16	23/14	80	Negative	Positive/Boryong (HQ660211)/ 118 d
4	F/89	Hypertension	Doxycycline, 10 d	10 d	+/+/+	7,200	150	48	311/225	160	ND	ND
				2.5 m	None	5,200	1	106	21/14	320	Positive	Positive/Boryong (HQ660201)/79 d
				4 m	None	3,900	Negative	61	27/22	40	Positive	Positive/Boryong (HQ660202)/ 104 d
5	F/31	Microscopic hematuria, thyroid cancer	Azithromycin, 10 d	6 d	+/-/-; pneumonia	6,600	48	21	17/19	80	Positive	Positive/Karp (HQ660203)/119 d
				4 m	None	9,600	4	25	10/6	160	Negative	Positive/Karp (HQ660204)/193 d
6	M/62	Hypertension, coronary heart disease, cerebral infarction	Doxycycline, 10 d	12 d	+/+/+	4,400	53	12	108/156	320	ND	Positive/Boryong (HQ660212)/ no record
				18 m	None	ND	0.8	12	29/34	160	Negative	Positive/Boryong (HQ660213)/ 200 d

+/-, present/absent; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; d, days; ESR, erythrocyte sedimentation rate; m, months; ND, not done; PCR, polymerase chain reaction.

CT, USA). The mixture was incubated in the thermal cycler at 94°C for 5 min, then cycled 30 times at 94°C for 1 min, at 55°C for 1.5 min, at 72°C for 2 min, and finally once at 72°C for 7 min. Amplification of the second half of the TSA56 gene was performed as described above except that primers 3 and 4 were used and the annealing period was shortened to 1 min instead of 1.5 min. The amplified products were electrophoresed in 1.5% agarose gels, purified with the QIAquick kit (QIAGEN), and sequenced by the single-pass sequencing method using the BigDye terminator cycle sequencing reaction kit and ABI PRISM 3700 & 3730xl automatic sequencer (Applied Biosystems, Foster City, CA, USA). All sequencing reactions were performed at least twice. Genotypes of the isolates were determined by identifying a representative strain revealing a maximum pairwise identity score with the TSA56 gene from the GenBank (National Institutes of Health, Bethesda, MD, USA), and sequence homology of the isolates were examined by pairwise comparisons using BLAST (National Institutes of Health).

Ethics statement

The study protocol was approved by the institutional review board of Inha University Hospital (IRB No. 11-1839). Informed consent was confirmed by the board.

RESULTS

All six patients revealed *O. tsutsugamushi* in blood culture in the afebrile state at 1 to 18 months after scrub typhus (Table 1). The genotypes of isolates were the Boryong in five patients (patients 2, 3, 4, and 6) and non-Boryong genotypes in two patients (Yonchon from patient 1 and Karp from patient 5). Nucleotide sequences of isolates serially collected from each patient were identical in all five patients in whom nucleotide sequences were compared. Nucleotide sequences of 9 isolates typed as the Boryong were 99% to 100% homologous, thus it is less helpful to differentiate each other; however, 2 isolates of the Yonchon and 2 isolates of the Karp types were distinctively different from other isolates. From 5 healthy persons and 5 patients with unrelated illnesses, no organism was isolated.

Patient 1, who presented with shock and was managed with steroid in addition to azithromycin, experienced a relapse 2 days after completion of 7-days' azithromycin therapy; treatment with doxycycline abated fever. Patients 1 and 2 complained of weakness in the absence of fever for 1 to 2.5 months after the illness and revealed positive blood nested PCRs; patient 1 showed abnormal aminotransferase level on day 31; and patient 2 revealed leukopenia on day 73, and retreatment with doxycycline resulted in improvement of weakness over 5 days. Patient 4 showed elevated erythrocyte sedimentation rate and positive PCRs 2 times over 4 months and experienced a transient ischemic attack (TIA) 8 months after scrub typhus. Patient 6, who had been managed

with angina pectoris, experienced dizziness of cardiac origin and underwent percutaneous coronary angioplasty 6 months after scrub typhus.

DISCUSSION

Asymptomatic harboring of *O. tsutsugamushi* or its DNA is frequently observed in animals, and these data are regarded as to represent an indirect evidence of persistent *O. tsutsugamushi* infection. For example, Jackson et al. (8) isolated *O. tsutsugamushi* from 12 (13.2%) among 91 wild rodents in Korea. Using PCR, *O. tsutsugamushi* DNA was detected in 12 (9.5%) among 127 wild rodents (9). In studies with experimentally infected mice, persistence of *O. tsutsugamushi* was more clearly demonstrated (10-13). Using mouse inoculation, persistence of viable *O. tsutsugamushi* is documented for up to 100 (10), 270 (11), and 610 days (12), irrespective of antibiotic therapy. Nested PCR also revealed persistence of *O. tsutsugamushi* DNA until 64 days after infection (13).

On the contrary, there have been few studies on the persistence of *O. tsutsugamushi* in human. Hayashi and Watanabe (5) detected *O. tsutsugamushi* bacteremia in a patient who was at 34 days after defervescence. The Pescadores strain of *O. tsutsugamushi* was isolated from a patient at 5 months after scrub typhus (6). Smadel et al. (7) recovered *O. tsutsugamushi* from the lymph nodes of 1 person among 12 who had experienced scrub typhus 1 to 2 yr previously. Because these 3 reports were performed before the introduction of modern technology, identification of isolates is not sufficient to affirm the homology of isolates. In volunteers experimentally infected by laboratory-reared chiggers, rickettsemia was present until 22 days after the infection (20). Thereafter, attempts to isolate viable *O. tsutsugamushi* from patients have seldom been tried.

Instead, in recent years, PCR has been used to show that *O. tsutsugamushi* DNA disappeared gradually over 5 to 27 days after administration of antibiotics and reaffirmed short duration of *O. tsutsugamushi* bacteremia in human (1-4). These PCR data were contrasted to the result of the present study, and the main reason for the difference is thought to be low sensitivity of PCR. Nested PCR is very sensitive enough to detect 10 attogram (10^{-18} g), i.e., 1 copy, of *O. tsutsugamushi* DNA, if it is performed by an experienced person and using Southern blot hybridization. However, if agarose gel electrophoresis is used, which was used in the above PCR studies, the sensitivity is lowered by 10^3 . Additionally, if clinical specimens such as blood or spleen are examined, it can lower the sensitivity of PCR further (13). Among the above PCR studies, only 1 study described that the detection threshold of the study method was 100 femtogram (10^{-15} g), i.e., 10^4 copies, of *O. tsutsugamushi* DNA (2). The quantity of *O. tsutsugamushi* in blood during scrub typhus, which was evaluated by real-time PCR, varies from undetectable level during days

1-4 to up to 10^6 copies per mL during days 9-12; and the detection limit of the real-time PCR was 1,062 target gene copies per mL of EDTA blood (21). We assume that the quantity of *O. tsutsugamushi* in blood during the chronic phase is below the quantity during days 1 to 4. Thus, nested PCR employed in clinical practice cannot detect *O. tsutsugamushi* DNA during the chronic phase in most scrub typhus patients. More importantly, the reason that the PCR studies did not detect *O. tsutsugamushi* DNA beyond 1 month of scrub typhus is that there has been little attempt to detect DNA beyond 1 month after the onset of scrub typhus (1-4, 21). Our study confirmed that the remaining DNA beyond the symptomatic period of scrub typhus was due to viable *O. tsutsugamushi*.

We used in vitro culture technique in addition to PCR. Cell culture has several disadvantages, such as requirement of long incubation, infectious hazard, and inability to detect dead organism; however, theoretically it can detect even 1 viable organism in blood. Regardless of which method is more sensitive, our study revealed a high isolation rate of *O. tsutsugamushi* during the chronic phase. One concern is laboratory contamination of cell culture during long incubation; however, this possibility can be excluded by the following reasons: firstly, our laboratory has about two decades' experiences in cultivating *O. tsutsugamushi* and maintains the contamination rate as minimal. Cell culture is not intrinsically prone to contamination as PCR, and if contamination occurs, bacterial contamination is more likely, and in turn culture is grossly spoiled. Secondly, nucleotide sequences of the isolates were identical within each patient, but different between patients infected by different genotypes. Lastly, culture from control persons did not revealed any organism.

Although the persistence of *O. tsutsugamushi* and its clinical significance in humans are firstly documented in the present study, persistence of *Rickettsia prowazekii*, a bacterium closely related to *O. tsutsugamushi*, in humans is well known, and Brill-Zinsser disease is a relapse of latent *R. prowazekii* infection. Likewise, persistent *O. tsutsugamushi* infection is associated with certain illnesses. It is already known that "early relapse", i.e., recurrence of fever within several days after completion of antibiotic therapy, is occasionally observed when antibiotic therapy is discontinued before the development of the protective immunity. In addition, "prolonged convalescence", i.e., persistent vague symptoms such as weakness in the absence of fever, as shown in patients 1 and 2, was clarified to be associated with the presence of *O. tsutsugamushi* in blood. This complication seems to occur as a result of slow development of the immunity. "Late relapse", i.e., recurrence of scrub typhus several months or years after the initial *O. tsutsugamushi* infection, is surely possible. Reactivation of dormant *O. tsutsugamushi* infection is documented in mice after inoculation of heterologous strain of *O. tsutsugamushi* or treatment with cyclophosphamide (14); in humans, coinfection of scrub typhus and leptospirosis (22), espe-

cially in patients not accompanied with eschar, may represent reactivation of scrub typhus triggered by leptospirosis.

Although it is not certain whether occurrence of the TIA in patient 4 and aggravation of coronary artery disease in patient 6 are related with persistent *O. tsutsugamushi* infection, these cases raise the necessity of long-term follow-up of scrub typhus patients, especially if they have multiple risk factors for atherosclerosis. *O. tsutsugamushi* is a representative bacterium infecting vascular endothelium, and infection may be one of risk factors for progression of atherosclerosis (23). Furthermore, antibiotics cannot eradicate *O. tsutsugamushi*, so once *O. tsutsugamushi* infection occurs, its effect may persists for a long time.

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