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ORIENTIA TSUTSUGAMUSHI IN PERIPHERAL WHITE BLOOD CELLS OF PATIENTS WITH ACUTE SCRUB TYPHUS

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Abstract. Scrub typhus, caused by *Orientia tsutsugamushi,* is an acute illness that occurs in many parts of Asia. Clinical manifestations range from inapparent to organ failure. Organisms disseminate from the skin to target organs, suggesting that they may enter the peripheral circulation. Here, peripheral blood cell smears from patients with acute scrub typhus were obtained before treatment and for 2 days after treatment and reacted with antibodies specific for *O. tsutsugamushi.* White blood cells from 3 of 7 patients with acute scrub typhus stained positively for *O. tsutsugamushi.* Cells containing *O. tsutsugamushi* were mononuclear and were detected on each day of sampling. The presence of *O. tsutsugamushi* in peripheral white blood cells of patients with acute scrub typhus is a new finding with clinical and pathogenic implications.

INTRODUCTION

Orientia (formerly *Rickettsia*) *tsutsugamushi,* the causative agent of scrub typhus, is a gram-negative obligate intracellular bacillus.[1] *Orientia tsutsugamushi* is transmitted during the bite of trombiculid mites, or chiggers, and leads to an infection that can range in severity from inapparent to fatal. In ~ 60% of primary infections, and less commonly in secondary infections, a cigarette burn-like lesion called an eschar develops at the site of the chigger bite. By analogy with Rocky Mountain spotted fever, endothelial cells are considered the key target cells.[2–5] However, it is not known whether organisms deposited in the skin spread to internal organs via the circulation, the lymphatics, as naked bacilli, or by another mechanism. A standard method for isolating *O. tsutsugamushi* from infected humans is to inoculate peripheral blood into mice at the bedside,[6] implying that organisms are present in the circulation. We therefore conducted a search for *O. tsutsugamushi* in circulating cells of patients diagnosed with acute scrub typhus.

MATERIALS AND METHODS

Participants. Adults presenting to Chiang Rai Regional Hospital, northern Thailand, with undiagnosed febrile illnesses of 1 week's duration or less were evaluated in conjunction with ongoing investigations of interactions between human immunodeficiency virus type 1 (HIV-1) and common tropical infections.[7] After informed consent was obtained, peripheral venous blood was collected from 7 patients with *O. tsutsugamushi* infection, confirmed by dot blot immunoassay.[8] All patients had uncomplicated disease. Specimens were examined before treatment, and again after 1 and 2 days of therapy with doxycycline.

Immunoalkaline phosphatase (AP) and direct immunofluorescent (DIF) staining. Heparinized blood was centrifuged at room temperature; buffy coats were harvested and 10–20-μL aliquots were placed on poly-L-lysine-coated or positively charged slides in a circular area of ~ 0.8 cm^2, air dried for 30–45 min, fixed with 1% formaldehyde and 1% Nonidet P40 (Boehringer Mannheim, Indianapolis, IN) for 10 min each, and then stored at 4°C until stained.

For AP staining, smears were reacted with a blocking solution containing 5% skim milk and 20% normal goat serum, followed by the primary antibody (polyclonal mouse antibodies specific for *O. tsutsugamushi,* Karp strain) diluted 1:250 with phosphate-buffered saline (PBS) (pH 7.4) containing 40% normal human serum.[9] Smears were then reacted for 30 min with the secondary antibody, alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Sigma Chemical, St. Louis, MO) diluted 1:100 in PBS containing 10% normal human serum, followed by application of naphthol AS-B1 phosphate/hexazotized new fuchsin, and levamisole (HistoMark Red; Kirkegaard Laboratories, Gaithersburg, MD) for 30 min as the chromogenic substrate. The smears were counterstained with Mayer hematoxylin for 1 min and then mounted with Immu-mount (Shandon, Pittsburgh, PA). The antigen was identified by scarlet red granules.

For DIF staining, buffy coats were harvested and fixed as described above. Cell smears were reacted for 60 min with fluorescein isothiocyanate (FITC)-conjugated polyclonal mouse antibodies specific for *O. tsutsugamushi* (Karp, Kato, and Gilliam strains) diluted 1:4 with PBS containing 40% normal human serum, and mounted with a 90% glycerol solution containing phenylenediamine (0.5 μL/mL). For double staining, selected smears were reacted with anti-*O. tsutsugamushi* FITC-conjugated antibodies overnight at 4°C, followed by phycoerythrin-conjugated anti-CD3 (PharMingen, San Diego, CA; specific for T cells) for 60 min. The slides were read with a Leitz epifluorescent microscope equipped with FITC and phycoerythrin filters. The *O. tsutsugamushi* antigen was identified by apple-green fluorescent granules, and the CD3-positive cells were orange-red.

Except for the double staining procedure, all AP and DIF steps were conducted at 37°C. After each staining step, slides were washed 3 times with PBS-Tween for 3 min at room temperature. For all experiments, smears of egg yolk sacs of developing chicken embryos infected with the *O. tsutsugamushi* (Karp strain) were used as a positive control; for negative control, we used peripheral blood mononuclear cell smears from patients with dengue fever, patients with fever of unknown origin, and healthy adult volunteers processed exactly as the buffy coats. Buffy coat smears obtained from a patient with *Plasmodium vivax* malaria served as an additional negative control.

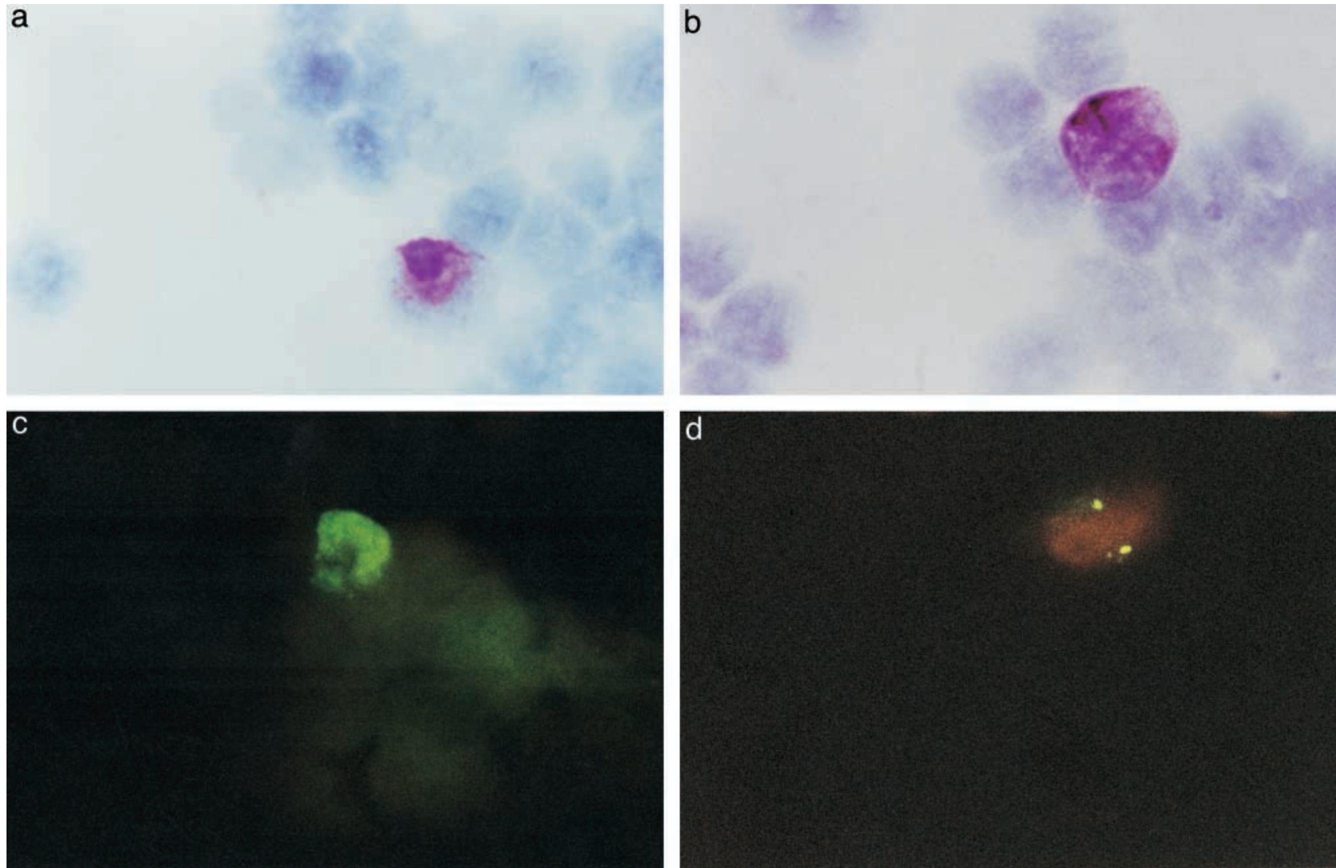

FIGURE 1. Peripheral blood smears from a patient with acute scrub typhus showing white blood cells containing *Orientia tsutsugamushi* (original magnification, ×1,000). **a, b**: stained by an immunoalkaline phosphatase method; *O. tsutsugamushi*-containing cells are depicted by scarlet red granules. **c, d**: stained by direct immunofluorescence; *O. tsutsugamushi*-containing cells are depicted by apple-green fluorescent granules.

RESULTS

By the AP method, *O. tsutsugamushi* was detected in buffy coat cells from 3 of 7 patients diagnosed with acute scrub typhus. Positive cells were detected in all 3 patients on Days 0–2, and the number of positive cells per smear ranged 1–10. Diffuse and focal cytoplasmic staining patterns were noted (Figure 1a, b). Immunoalkaline phosphatase-positive cells were mononuclear, many with monocytic features. No AP-positive polymorphonuclear cells were detected. In 7 negative control smears (3 dengue fever, 3 fever of unknown origin, and 1 vivax malaria), one fever of unknown origin smear contained several positively staining cells with lymphocytelike morphology. Scrub typhus serology from this subject was negative. The other 6 smears were negative. Ten peripheral blood mononuclear cell smears from healthy adults were AP negative (specificity 100%).

For DIF, one AP-positive scrub typhus sample was selected for testing. Similar to AP staining, there were positive cells with focal and diffuse staining (Figure 1c, d). None of the *O. tsutsugamushi* positive cells were CD3 positive. A random selection of negative control samples did not react with the anti-*O. tsutsugamushi* antibodies (specificity 100%).

DISCUSSION

Scrub typhus, acquired when *O. tsutsugamushi* organisms are deposited in the skin by the bite of an infected trombiculid mite, may remain unnoticed or progress to a potentially fatal disease usually involving the lungs.[10] The mechanism of dissemination from the skin to internal organs is not known, but experimental models suggest organisms travel from the skin to regional lymph nodes, then spread to target organs.[1] Organisms can be isolated by injecting mice with blood from infected patients, implying that they enter circulation. Here, we provide what is to our knowledge the first evidence that *O. tsutsugamushi* is present in circulating white blood cells of humans with acute scrub typhus. Cells with *O. tsutsugamushi* were mononuclear, many with monocytic features, and displayed staining characteristics similar to experimentally infected cells.[1]

Scrub typhus is most often diagnosed by Weil-Felix test, but this assay lacks sensitivity.[11–13] Improved serodiagnostic methods are not generally available in endemic areas.[11] Direct visualization of organisms on a peripheral smear has potential as a diagnostic test. The reappearance of *O. tsutsugamushi* during therapy could indicate treatment failure, a concern in areas where drug-resistant strains of scrub typhus occur.[14,15]

In patients with HIV-1, coinfection with *O. tsutsugamushi* suppresses viral replication, but the mechanism of suppression has not been elucidated.[7] Both HIV-1 and *O. tsutsugamushi* are obligate intracellular organisms with similar life cycles.[16] The detection of *O. tsutsugamushi* in mononuclear cells raises the issue of whether HIV-1 inhibition may be

related to the invasion of the same cells by *O. tsutsugamushi* and HIV-1.

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