IMMUNOHISTOCHEMICAL STUDY OF SCRUB TYPHUS: A REPORT OF TWO CASES

Bo-Yuan Tseng,1 Hui-Hua Yang,2 Ji-Hung Liou,1 Li-Kuang Chen,3 and Yung-Hsiang Hsu1
Departments of 1Pathology, 2Virology and 3Emergency Medicine, Buddhist Tzu Chi General Hospital and University, Hualien, Taiwan.

Scrub typhus is a zoonotic disease caused by Orientia tsutsugamushi, which is transmitted by chiggers. It is widely distributed in Asia Pacific. Clinically, about 50% of patients present with an eschar at the site of attachment of the infected mite. The onset is a sudden high fever. Then, macular rash develops. In uncomplicated infections, the fever abates in about 2 weeks [1]. The basic pathologic lesion is vasculitis. Most fatal cases result from the development of myocarditis and rickettsial interstitial pneumonitis [2]. Identification of O. tsutsugamushi in tissue sections is a key to understanding the pathogenesis of this disease.

Recently, our group developed a specific antibody against O. tsutsugamushi, which was found to be suitable for immunohistochemical study on formalin-fixed and paraffin-embedded tissue sections. We collected tissue sections from two scrub typhus patients and performed a serial immunohistochemical study to investigate the distribution of O. tsutsugamushi in human tissue.

CASE PRESENTATIONS

Case 1
One week prior to presentation, a 42-year-old man, a prisoner of Green Island, felt epigastralgia. High fever, nausea, vomiting and diarrhea also developed on the night of August 22, 2002. He was sent to Taitung Army 805 Hospital on the morning of August 23. The only medicine administered was to relieve the gastrointestinal symptoms. He was sent to the intensive care unit (ICU) at 14:10 PM when he was found unconscious. Apnea occurred suddenly, 2 minutes after ICU admission. Cardiac pulmonary resuscitation was carried out immediately, but in vain, and he died at 14:40 PM.

Autopsy revealed vasculitis and perivasculitis in the heart and brain, interstitial myocarditis and interstitial pneumonitis with alveolar edema and hemorrhage, which were the cause of death. No eschar lesions were found on the body.
Case 2
An 8-year-old girl received an insect bite on the left upper scapula with eschar formation. After 2 weeks, she experienced high fever with headache on July 25, 2005, and she visited a local medical doctor. She was transferred to Guanshan Tzu Chi Hospital due to intermittent fever and severe abdominal pain accompanied by vomiting on July 31. An appendectomy was performed on August 1. She was then transferred to Hualien Tzu Chi Hospital due to postoperative persistent fever on August 2. A sudden drop in blood pressure (60/40 mmHg), severe dyspnea and cyanosis developed at 3:00 AM on August 3. She was transferred to the pediatric ICU due to acute respiratory distress syndrome (ARDS). She died of respiratory failure on August 4 at 1:00 AM. *O. tsutsugamushi* was isolated from premortem blood.

An autopsy revealed vasculitis and perivasculitis in the skin, heart and brain, interstitial myocarditis and interstitial nephritis, aseptic meningoencephalitis, and interstitial pneumonitis with hyaline membrane formation, which were the cause of death. In addition, hemophagocytosis was observed in bone marrow, multiple lymph nodes, liver and spleen.

Methods

Preparation of monoclonal antibodies to *O. tsutsugamushi* antigen

*O. tsutsugamushi* were harvested 5–7 days after growth in L-cells. Six- to 8-week-old BALB/c mice were intraperitoneally or subcutaneously injected with *O. tsutsugamushi*. When the anti-*O. tsutsugamushi* titters rose, mice were sacrificed and spleen cells were fused with myeloma cells. The supernatants of hybridoma cultured media were screened with *O. tsutsugamushi* antigens by indirect immunofluorescent assay. Ascites fluid was collected from SCID mice intraperitoneally injected with *O. tsutsugamushi*. When the anti-*O. tsutsugamushi* titters rose, mice were sacrificed and spleen cells were fused with myeloma cells. The supernatants of hybridoma cultured media were screened with *O. tsutsugamushi* antigens by indirect immunofluorescent assay. Ascites fluid was collected from SCID mice intraperitoneally injected with *O. tsutsugamushi*. The monoclonal antibody-recognized 47-kDa component of *O. tsutsugamushi* was placed on the specimens at a dilution of 1:100 with DAKO diluent made

Specimens

Autopsy specimens and a surgical specimen (appendix, Case 2) were archived as formalin-fixed, paraffin-embedded tissue blocks at the Department of Pathology, Tzu Chi Hospital. Paraffin sections of 4 μm in thickness were cut from blocks of heart, lung, liver, kidney and brain from Case 1 and heart, lung, liver, spleen, kidney, brain, appendix and skin from Case 2.

Immunohistochemical staining

The sections of tissue specimens were placed in poly-L-lysine, silane-coated slides and incubated at 70°C for 20 minutes. They were rehydrated in water and digested with antigen retrieval solution (DAKO, Carpinteria, CA, USA) for 20 minutes at 99°C. The slides were incubated in 3% aqueous hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity and rinsed in deionized water. Nonspecific binding of antibody was blocked by incubating specimens with normal goat serum and avidin blocking reagent (Vector Laboratories, Burlingame, CA, USA), which had been mixed (1:10) for 30 minutes.

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up with biotin blocking reagent and incubated at 4°C overnight. This step was followed by successive incubations with biotinylated second anti-mouse IgG (1:800) (DAKO; half hour) and a tertiary streptavidin–peroxidase (DAKO; half hour). Color development was obtained by incubation for 5 minutes with AEC [5]. The sections were counterstained with hematoxylin, dehydrated and mounted in glycerol. Positive and negative control sections were run in parallel with each assay. We used one eschar lesion as a positive control, which had been proved to be scrub typhus at the US Centers for Disease Control in 2001. Samples were considered positive for scrub typhus when distinctive cells revealed granular cytoplasmic deposits.

RESULTS

Immunohistochemical localization of *O. tsutsugamushi*

The basic histopathologic lesions in the human autopsy specimens were disseminated mononuclear perivasculitis, interstitial nephritis and interstitial myocarditis, with one of the most important lesions being interstitial pneumonitis with alveolar edema. Immunohistochemical staining of all specimens detected *O. tsutsugamushi* in endothelial cells of the organs evaluated, including many blood vessels (Figure 2). *O. tsutsugamushi* was also detected within macrophages from the lymph node and spleen samples (Figure 3). An unexpected finding was the identification of intracellular bacteria within cardiac muscle fibers (Figure 4A) and renal tubules (Figure 4B).

DISCUSSION

At least eight antigens in three strains of *O. tsutsugamushi* have been found. They express epitopes of the 150 kDa, 110 kDa, 72 kDa, 58 kDa, 56 kDa, 49 kDa, 47 kDa and 20 kDa polypeptide antigens [6]. The principal cell wall constituents of *O. tsutsugamushi* comprise the major strain variable 56-kDa protein as well as the antigenically variable 110-kDa, 47-kDa and 20-kDa proteins [6–10]. We developed a monoclonal antibody recognizing the 47 kDa antigen, which is a surface protein of *O. tsutsugamushi*. The function was unknown. This antibody is specific for *O. tsutsugamushi*. We also developed another monoclonal antibody against the 56 kDa protein (unpublished data). However, the absence of lipopolysaccharide in *O. tsutsugamushi* and the antigenic variation of the 56-kDa major surface protein make the detection of this organism a special challenge [7,11]. Thus, we selected the monoclonal antibody against the 47-kDa protein for this study.

*O. tsutsugamushi* was distributed in the endothelial cells of nearly all representative organs, similar to the distribution noted by Moron et al [12], especially in the eschar lesion in Case 2. Direct detection of rickettsia in eschars would allow for prompt diagnosis and treatment without waiting for patient antibodies to develop. *O. tsutsugamushi* was unexpectedly found in the endothelial cells of the appendix in Case 2. This is a cause of acute abdominal pain [13], such as that seen in our cases. To our knowledge, this is the first report to describe this organism in the gastrointestinal tract.

In addition to endothelial cells, we also found that *O. tsutsugamushi* may distribute in the reticuloendothelial system, such as macrophages of liver, spleen, lymph node and bone marrow. Macrophage activation-induced hemophagocytosis may develop, as shown in Case 2 [14,15].

Host protection against rickettsial infection has been found to consist of a complex interaction between cell-mediated and humoral factors [16]. Peak macrophage activation has been found to occur during the active process of rickettsial clearance [15]. The details of the immunologic reaction against rickettsial infection, including activation of cytokines, are not fully understood, but macrophages and T-lymphoid cells are believed to play important roles in preventing rickettsial infection and the appearance of hemophagocytosis syndrome [17]. Hemophagocytosis syndrome may represent a hyper-reaction of the immune system mediated by an accelerated cytokine network during the advanced stages of this disease [15].

It is of note that *O. tsutsugamushi* may be found in cardiac cells and tubular epithelium, and this microorganism may directly injure such organs resulting in myocardial necrosis [12,18] and acute tubular necrosis with acute renal failure [19]. To our knowledge, for the first time, this organism has been identified within the renal tubules.

Diffuse alveolar damage with hyaline membrane formation was the cause of death in both cases. Immunohistochemistry staining showed only some endothelial cells positive for *O. tsutsugamushi*. It is
difficult for us to explain the pathogenesis. Scrub typhus associated with ARDS due to a cytokine storm as part of the immune response to *O. tsutsugamushi* infection is highly suspected.

*O. tsutsugamushi* was transferred to lung tissue via endothelial cells or macrophages. Interstitial pneumonia with lymphocytic infiltration developed. Inducible nitric oxide synthase (iNOS) is induced by a number of inflammatory cytokines and mediators, most notably interleukin-1, tumor necrosis factor and interferon-γ. Human alveolar macrophages and monocytes are a source of nitric oxide (NO).
production [20]. The iNOS produced a large amount of NO. High NO concentrations are cytotoxic because NO is an unstable molecule that triggers the formation of oxidative free radicals such as peroxynitrite (ONOO–) and dinitrogen trioxide [21]. At higher levels, these short-lived molecules are implicated in a variety of tissue injury mechanisms, including (1) endothelial damage with thrombosis and increased permeability; (2) protease activation and antiprotease inactivation, with a net increase in breakdown of the extracellular matrix; and (3) direct injury to parenchymal cells such as alveolar cells [22]. Finally, ARDS develops.

In conclusion, O. tsutsugamushi may disseminate into multiple visceral organs via endothelial cells and macrophages and result in fatal complications.

REFERENCES

以免疫組織化學染色法偵測恙蟲病病人組織內立克次體分佈之研究 — 两病例報告

曾柏元¹ 楊惠華² 劉嘉鴻¹ 陳立光³ 許永祥¹
花蓮慈濟醫院暨慈濟大學 ¹病理科 ²病毒室 ³急診科

恙蟲病病原是立克次體經由沙蚤媒介之人畜共通傳染病。此種立克次體在人類的標的細胞目前仍未釐清，免疫組織化學染色應用在恙蟲病病人組織切片上可釐清這個立克次體的標的細胞，我們用 47KD 專一對抗立克次體的抗體將二例解剖標本作免疫組織化學染色，染色結果顯示立克次體分佈在所有標本包括心臟、肺臟、腎臟、腸尾及皮膚的內皮細胞和腎臟腎小管上皮細胞、心肌細胞及淋巴腺、肝臟、脾臟內的吞噬細胞。恙蟲病立克次體可經由內皮細胞及吞噬細胞擴散到多個器官而造成致死性的併發症。

關鍵詞：免疫組織化學染色，日本秋恙蟲，叢林型斑疹傷寒
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