Identification of the Target Cells of *Orientia tsutsugamushi* in Human Cases of Scrub Typhus

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***Orientia tsutsugamushi* is the etiologic agent of scrub typhus, a chigger-borne zoonosis that is a highly prevalent, life-threatening illness of greatest public health importance in tropical Asia and the islands of the western Pacific Ocean. The target cell of this bacterium is poorly defined in humans. In this study, *O. tsutsugamushi* were identified by immunohistochemistry using a rabbit polyclonal antibody raised against *O. tsutsugamushi* Karp strain in paraffin-embedded archived autopsy tissues of three patients with clinical suspicion of scrub typhus who died during World War II and the Vietnam War. Rickettsiae were located in endothelial cells in all of the organs evaluated, namely heart, lung, brain, kidney, pancreas, and skin, and within cardiac muscle cells and in macrophages located in liver and spleen. Electron microscopy confirmed the location of rickettsiae in endothelium and cardiac myocytes.**

KEY WORDS: Immunohistochemistry, *Orientia tsutsugamushi,* Rickettsia, Scrub typhus.

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Scrub typhus is a chigger-borne zoonosis that is of greatest public health importance in tropical Asia and the islands of the western Pacific Ocean. It became known to the West during World War II, when large numbers of cases occurred in soldiers of both sides in the Pacific and Asian theater. Outbreaks were associated with the early stage of development of a camp when chiggers were plentiful, with variable case-fatality rates ranging from <1% to >30% (1, 2). Among indigenous populations, it is a frequent cause of febrile illness leading to admission to a hospital (3). *Orientia* (formerly *Rickettsia*) *tsutsugamushi,* the etiologic agent of scrub typhus, is maintained in nature by highly efficient transovarial transmission in trombiculid mites and is transmitted to humans by the larval mite (chigger) during feeding. Although *O. tsutsugamushi,* a member of the family *Rickettsiaceae,* is phylogenetically related to the genus *Rickettsia* with 90.2 to 90.6% homology by 16S rRNA gene sequences, its cell wall construction differs substantially and is distinguishable ultrastructurally (4–6). Although the cell wall of *Rickettsia* contains abundant, immunodominant lipopolysaccharides, outer membrane protein B, a 17-kDa lipoprotein, and, for spotted fever group rickettsiae, outer membrane protein A, *O. tsutsugamushi* contains nothing even analogous to these components. The principal cell wall constituents of *O. tsutsugamushi* are the major strain-variable 56-kDa protein as well as antigenically variable 110-, 47-, and 25-kDa proteins (7–11).

The pathogenesis of scrub typhus is even less well defined than that of the spotted fever and typhus group rickettsioses. Between 1916 and 1922, Wolbach and colleagues (12, 13) developed and applied modifications of the Giemsa stain to the demonstration of *R. rickettsii* and *R. prowazekii,* detecting intracellular rickettsiae in endothelial cells of cutaneous biopsies and necropsies of patients with Rocky Mountain spotted fever and epidemic louse-borne typhus, respectively. However, the target cell or cells of *Orientia tsutsugamushi* are essentially undefined. In a study of 78 autopsies of cases of scrub typhus, Allen and Spitz (14) evaluated all the tissues of six appropriately fixed cases by Wolbach's modification of the Giemsa stain and detected rickettsiae in endothelial cells of only one site, an eschar, in a single case and in macrophages of a smear of spleen in a single case. Settle *et al.* (15) reported the demonstration of orientiae only in smears of human pericardial, pleural, and peritoneal fluid. It is difficult to understand the pathology and pathophysiology of a disease for which the

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locations of the infection in the organs are unknown, particularly for an obligately intracellular infectious agent. Thus, using immunohistochemistry (IHC) and electron microscopy, this investigation was undertaken to detect *O. tsutsugamushi* in formalin-fixed, paraffin-embedded autopsy tissues from three U.S. Army soldiers who died with clinical suspicion of scrub typhus during World War II and the Vietnam War, as well as in experimentally inoculated animals.

CASE REPORTS

Case 1

On November 7, 1943, generalized myalgia, malaise, headache, and enteritis developed in this 35-year-old white male soldier serving in New Guinea. On the second day of illness, before presentation for medical attention, he noticed an eschar on the right upper arm that was 1 cm in diameter, with a black, necrotic ulceration and peripheral edema. He was admitted to the hospital, where physical examination revealed tender right axillary lymphadenopathy and an erythematous macular rash over the chest and abdomen. On Day 10 of illness, he showed disorientation, hyperreflexia, generalized lymphadenopathy, and a rising fever. On Day 13, the patient became completely irrational, there was generalized hypertonicity of all muscle groups, early Cheyne-Stokes breathing, blood pressure of 105/62 mm Hg, pulse at 120 beats/minutes, and a soft systolic murmur over the mitral area. Later that day he developed ileus. The next day, he became increasingly dyspneic and tachycardic, developed a generalized clonic seizure, and died. Necropsy revealed vasculitis and perivasculitis in heart, brain, and kidneys; myocarditis; interstitial pneumonia with alveolar edema and hemorrhages; splenomegaly; and an eschar on the right upper arm. *O. tsutsugamushi* was isolated from postmortem cardiac blood by inoculation of mice.

Case 2

This 33-year-old soldier who had been working as a laborer on the roads in New Guinea was admitted to the hospital on February 24, 1945 complaining of having had malaise, anorexia, headache, and fever for 2 days. Physical examination revealed fever, a blood pressure of 94/60, and moderate conjunctival injection, and after admission, he developed a dry cough. On Day 9 of illness, rales were detected at the left base, the sputum showed pneumococci, the temperature was 103°F (39.4°C), and penicillin therapy was begun. On the 14th day of illness, the patient who had been lethargic seemed more alert, and his blood pressure was 120/60 mm Hg. Suddenly, he complained of nervousness, suffered a short, generalized, convulsive seizure, and expired. Necropsy revealed vasculitis and perivasculitis in heart, brain, and kidneys; interstitial myocarditis; severe interstitial pneumonitis with hemorrhages; splenic hemorrhages; hepatic necrosis with portal triaditis and perivasculitis; interstitial nephritis with multiple hemorrhages; and an eschar.

Case 3

This 21-year-old soldier was admitted to a hospital in Vietnam on September 28, 1965 with a 3-day illness with a prodrome of weakness, anorexia, and headache followed by chills and fever for 48 hours. On physical examination, his temperature was 105°F (40.5°C) and blood pressure, 110/70 mm Hg. He was alert and cooperative, and diffuse inspiratory and expiratory wheezes were heard throughout both lung fields. The rest of the examination was normal. On Day 4 of illness, he was diagnosed with falciparum malaria by blood smear. His illness did not respond to pyrimethamine treatment. A maculopapular rash was observed on the 6th day of illness. Oral quinine was initiated on Day 5 of illness and was changed to intravenous treatment 3 days later. He developed a bleeding tendency with coffee-ground emesis, an hematocrit of 40, white blood cells per cell count of 1000 cells per μ, bleeding time of 5 minutes, and absence of platelets on peripheral blood smear. He suffered deafness, tachypnea, dyspnea, and hematemesis, passed large bloody stools, became incontinent and very restless, and died on the 12th day of illness. During his course, his body weight increased from 135 to 145 pounds. Necropsy revealed bleeding in the lungs, stomach, bowel, brain, and diaphragm; splenomegaly; and hepatomegaly with fatty metamorphosis.

MATERIALS AND METHODS

Autopsy specimens were archived as formalin-fixed, paraffin-embedded tissue blocks at the Armed Forces Institute of Pathology in Washington, D.C. Paraffin sections 4 μm in thickness were cut from blocks of heart, lung, spleen, liver, pancreas, kidney, and brain from Case 1; from lung, spleen, liver, kidney, and brain from Case 2; and from heart, lung, spleen, pancreas, kidney, brain, and skin from Case 3. Sections were also cut from blocks of heart, lung, liver, spleen, kidney, and testes from two Balb/c mice previously inoculated intraperitoneally with a tissue culture suspension of *O. tsutsugamushi,* one of them with Karp strain and the other with Gilliam strain. The mouse infected with Karp strain was sacrificed on Day 6 after inoculation, and the one infected with Gilliam strain, on

Day 8. We used a polyclonal antibody raised against *O. tsutsugamushi,* Karp strain, developed in the Rickettsial and Ehrlichial Diseases Research Laboratory, University of Texas Medical Branch, as follows: a rabbit was injected intraperitoneally with 1.0 mL of purified *O. tsutsugamushi*. After 1 month, a booster immunization was administered, and 10 days later, serum was obtained for antibody titration. The IFA titer of the serum was 1:1280.

The sections were placed in poly-L-lysine, silane-coated slides and incubated at 70°C for 20 minutes, then rehydrated in water and digested with antigen retrieval solution (DAKO, Carpinteria, CA) 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) mixture (1:10) for 30 minutes. The anti-*Orientia* antibody was placed on specimens at a dilution of 1:500 with DAKO diluent made up with biotin blocking reagent and incubated at room temperature for 30 minutes. This step was followed by successive 15-minute incubations with biotinylated anti-rabbit IgG (1:800) and the streptavidin reagent (DAKO, Carpinteria, CA). Color development was obtained by incubation for 5 minutes with diaminobenzidine (3,3'-diaminobenzidine). Sections were counterstained with hematoxylin, dehydrated, and mounted in Permount.

IHC controls consisted of the following: (1) substitution of normal rabbit serum for the primary antibody to rule out nonspecific binding; (2) substitution of anti–spotted fever group polyclonal antibody and monoclonal antibody to typhus group LPS for the primary antibody to rule out cross-reactions; (3) evaluation of human tissue known not to contain rickettsiae; and (4) evaluation of tissues of mice known to be infected with *Orientia* as positive controls.

An area of myocardium of Patient 1 with the presence of *O. tsutsugamushi* identified in histochemically stained slides was marked on the paraffin block from which the section was made. A piece of tissue was cut out from the rickettsiae-containing area, deparaffinized in xylene, fixed in 1% O_SO_4 in 0.1-M cacodylate buffer, stained *en bloc* in 2% aqueous uranyl acetate, dehydrated in ethanol, and embedded in PolyBed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut with a Reichert Ultracut S ultramicrotome (Leica, Deerfield, IL), stained with lead citrate, and examined in a Philips 201 electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) at 60 kV.

RESULTS

Immunohistochemical Localization of *O. tsutsugamushi*

The anti-*Orientia* immunohistochemical method demonstrated the bacteria in macrophages within the tissue sections from the mouse inoculated with Karp strain and also from that inoculated with Gilliam strain (Fig. 1). The rickettsiae were located within macrophages that had infiltrated the capsules of the liver and spleen as well as in splenic macrophages.

The basic histopathologic lesions in the human autopsy specimens were disseminated mononuclear perivasculitis, one of the most important lesions being interstitial pneumonia with hemorrhage and alveolar edema. The IHC staining of these specimens detected *Orientia* in endothelial cells of all of the organs evaluated, including in many blood vessels without perivascular mononuclear infiltrates (Fig. 2, A–E). *O. tsutsugamushi* was also detected within macrophages located in liver and spleen (Fig. 2, D, F). An unexpected finding was the identification of intracellular bacteria within well preserved cardiac muscle fibers in Cases 1 and 3 (Fig. 2E). The IHC assay showed no staining of *Orientia* when the primary antibody was replaced by normal rabbit serum or by anti-SFG or anti-typhus group rickettsial antibody. There was also no staining when the tissue

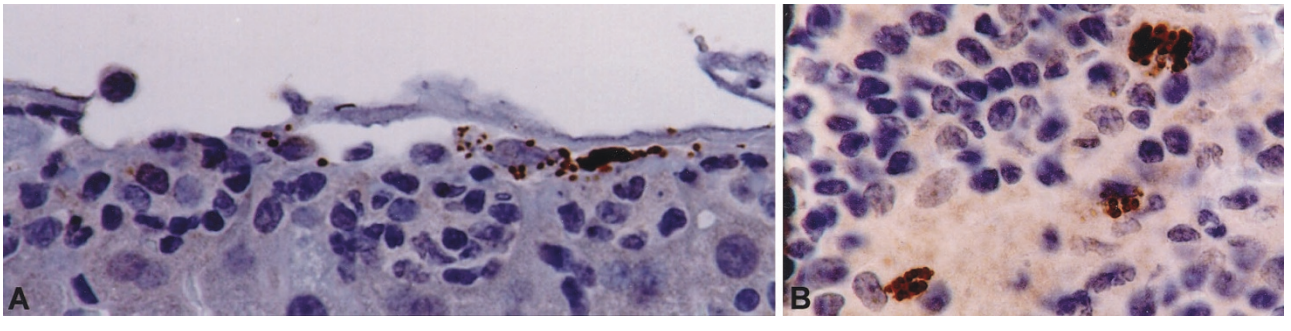

FIGURE 1. Immunohistochemically stained *O. tsutsugamushi* in macrophages in (**A**) the liver capsule (240×) and (**B**) the splenic parenchyma of an experimentally infected mouse 160×.

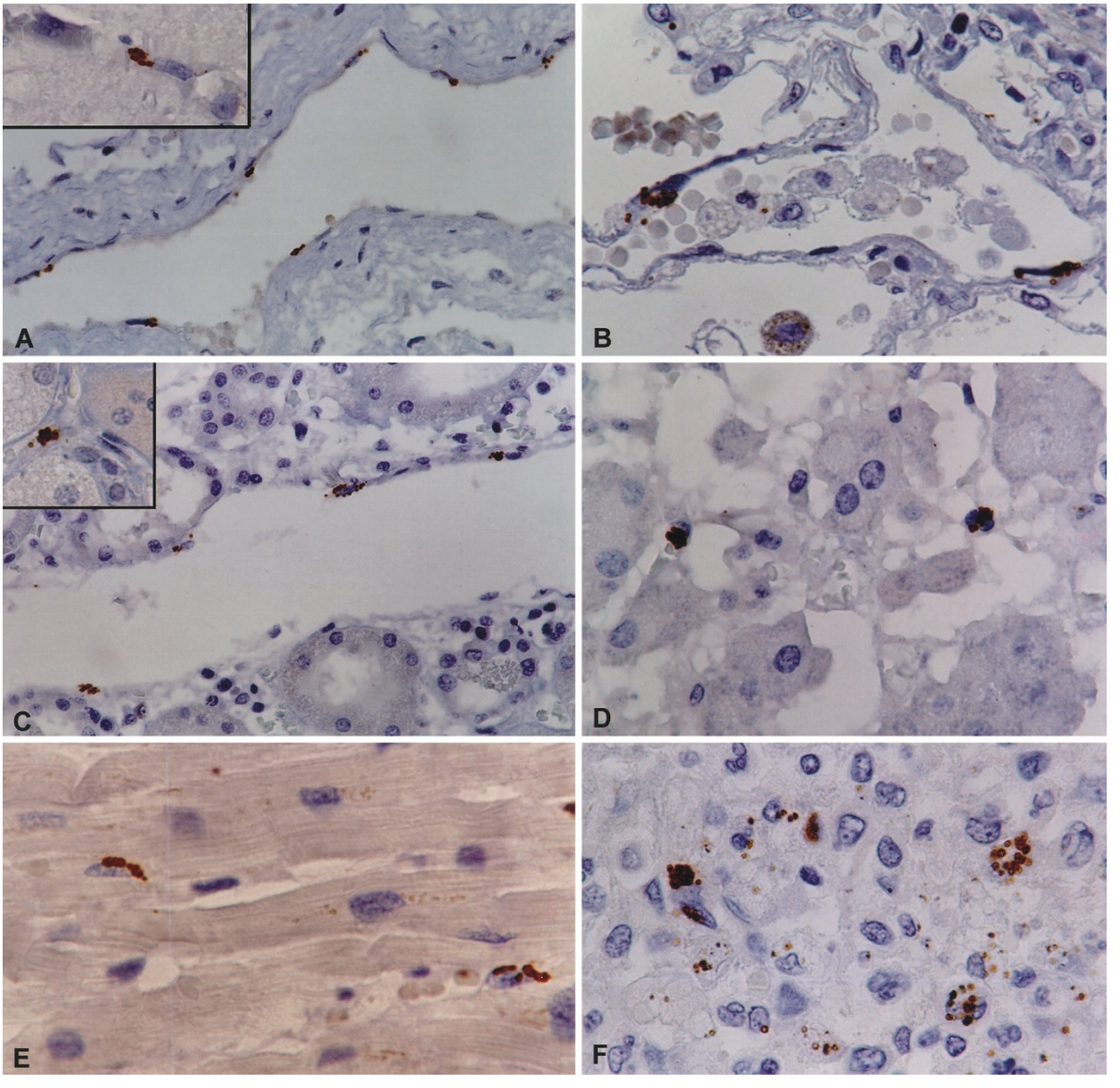

FIGURE 2. Immunohistochemically stained *O. tsutsugamushi* in endothelium in the brain of Case 2 (**A** [100×]; inset, 160×), in the lung of Case 3 (**B** [120×]), and kidney of Case 2 (**C** [100×]) and Case 1 (**C** inset [120×]). Liver of Case 3 showing antigen of *O. tsutsugamushi* in Kupffer cells (**D** [160×]). Spleen of Case 3 showing *O. tsutsugamushi* in macrophages in the red pulp (**F** [160×]). The heart of Case 3 showing location of *O. tsutsugamushi* in cardiac muscle cells and also in endothelium (**E** [160×]).

evaluated was human tissue not infected with *O. tsutsugamushi.*

Ultrastructural Observations

Orientia tsutsugamushi were found in endothelial cells of myocardial capillaries and in cardiac myocytes. Preservation of ultrastructural details in the sections was far from optimal as a result of formalin fixation and paraffin embedding, but *Orientia* were recognizable and identifiable inside the cells.

They were consistently found in the cytoplasm of capillary endothelial cells (Fig. 3A, arrows), which were observed to be heavily infected, containing several bacteria. A bacterium appeared to be in the process of exiting the host cell by budding (Fig. 3A, arrowhead). The budding rickettsia appeared to be surrounded by the plasma membrane of the host cell (Fig. 3, B–C, arrows). This mode of exit from the host cell is very characteristic of *Orientia* (4, 16). Another *Orientia*-specific feature was the thickness of the outer leaflet of the outer membrane of the cell wall (Fig. 3, C–D, arrowheads), which is thicker than the inner leaflet, contrary to what is observed in bacteria belonging to the genus *Rickettsia.* This ultrastructural feature is diagnostic for the genus *Orientia* (5). The rickettsial cytoplasmic membrane was generally poorly preserved in these samples but

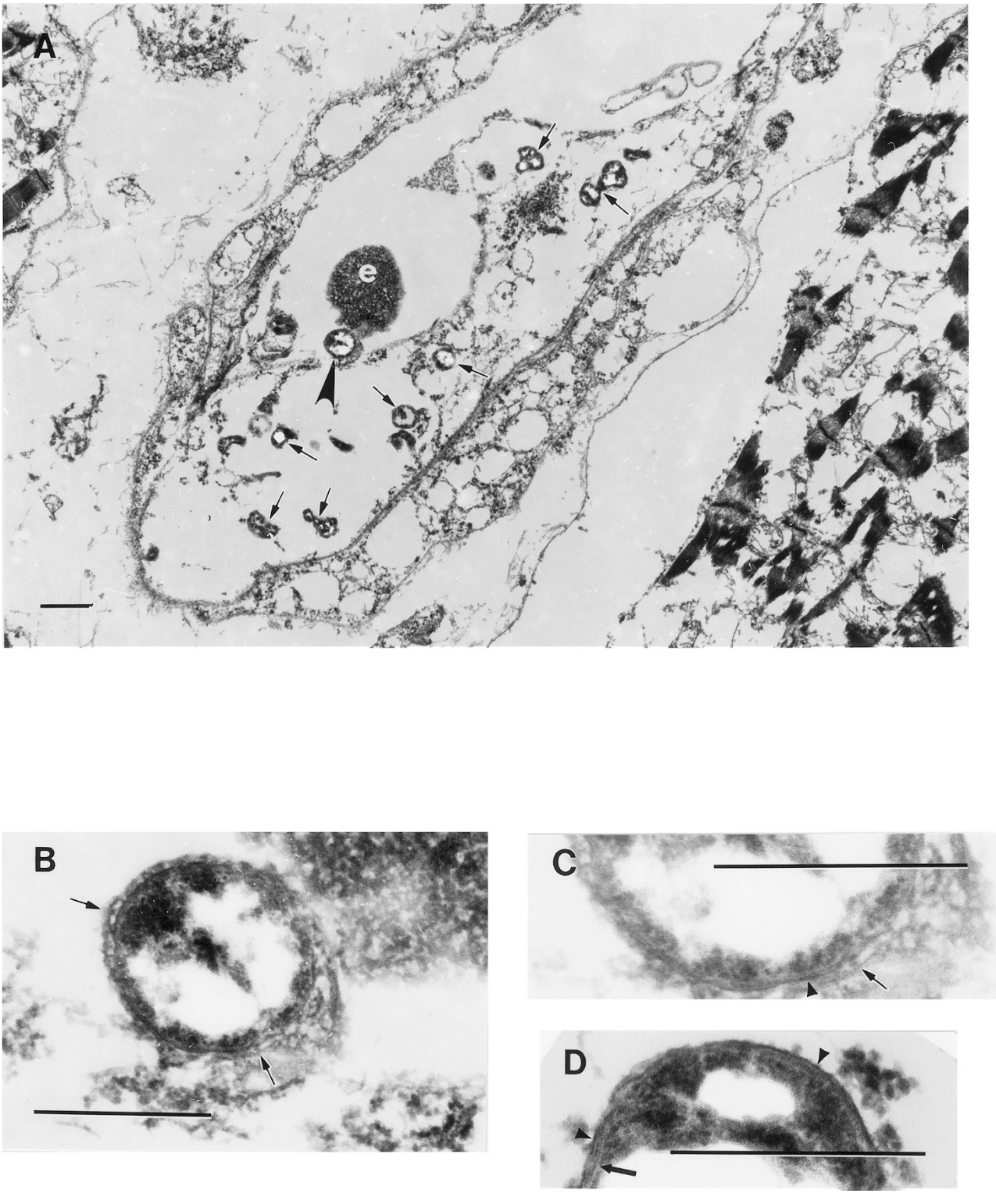

FIGURE 3. *O. tsutsugamushi* in an endothelial cell of a myocardial capillary. **A,** The capillary endothelial cell contains several bacteria in its cytoplasm (***arrows***). One bacterium appears to be budding from the endothelial cell surface (***arrowhead***). E-erythrocyte in the capillary lumen. Bar = 1 μm. **B,** Higher magnification of a budding *Orientia* cell, surrounded by the plasma membrane (***arrows***) of the endothelial cell. Bar = 0.5 μm. **C,** Part of the cell wall of the rickettsia shown in Figure 3B demonstrating a thicker outer leaflet of the cell wall (***arrowhead***). Arrow indicates host cell membrane surrounding the bacterium. Bar = 1 μm. **D,** Part of an envelope of an orientia in another endothelial cell showing thicker outer leaflet of the outer membrane of the cell wall (***arrowhead***) and a cytoplasmic membrane ((***arrow***). Bar = 1 μm.

could be identified in some organisms (Fig. 3D, arrow).

Bacteria with similar ultrastructure were also regularly found in the cytosol of cardiac myocytes, usually between the bundles of muscle filaments (Fig. 4, A–B, arrows). These organisms also had a typical thick outer leaflet of the outer membrane of the cell wall (Figure 4, C–D, arrowheads). They were

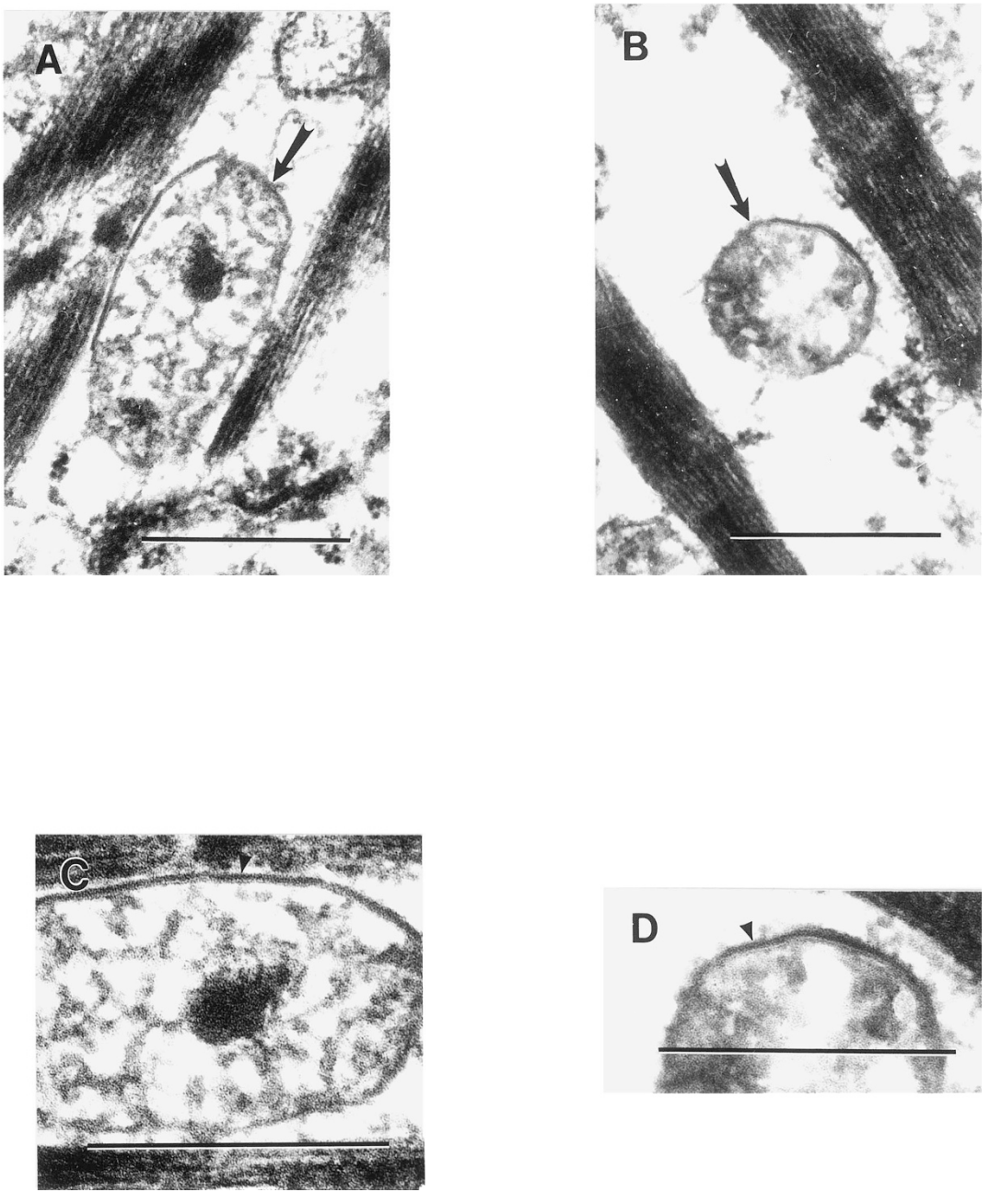

FIGURE 4. *O. tsutsugamushi* in the cardiac myocytes of patient 1. Bar = 0.5 μm. **A, B,** *Orientia* cells (***arrows***) in the cytosol lying between the myofibrils of cardiac myocytes. **C, D,** Higher magnification of *Orientia* cells showing the typical thicker outer leaflet of the cell wall (***arrowheads***).

usually located as single rickettsiae rather than in groups as in endothelial cells, and they were clearly distinguishable from mitochondria.

These ultrastructural data confirm the observations from immunohistochemically stained sections that *O. tsutsugamushi* can infect endothelial cells and cardiac myocytes in patients with scrub typhus.

DISCUSSION

The discovery that *O. tsutsugamushi* infects principally endothelial cells in all of the organs available for examination with smaller quantities of infected macrophages and cardiac myocytes is a substantial addition to our foundation of knowledge of scrub typhus. The fundamental pathophysiology of Rocky Mountain spotted fever is that of increased vascular permeability, edema, hypovolemia, and hypoperfusion of some organs such as the kidneys (17). Although the pathogenesis of scrub typhus may be very complex and involve immune and inflammatory mediators such as cytokines, prostaglandins, leukotrienes, and kinins, the key factor of disseminated endothelial infection has now been established for tsutsugamushi disease as well as the rick-

ettsioses. Indeed, Case 3 was apparently associated with generalized edema, and pleural, pericardial, and peritoneal effusions were reported in a substantial portion of fatal cases necropsied during World War II (15, 18). The histopathology of scrub typhus was investigated carefully in reports of 27, 31, 55, and 78 cases (14, 15, 18, 19). The consensus in 1945 was that disseminated vasculitis with perivasculitis was the paramount lesion, with involvement of the brain and lungs being the most important factors in a fatal outcome. Vascular damage was manifested prominently by hemorrhagic phenomena, but thrombi were notably quite rare. At that time, the issue of myocardial damage and potentially acute and chronic cardiac insufficiency owing to the histologically evident interstitial inflammation was of particular concern. The identification of *O. tsutsugamushi* in cardiac myocytes reopens this question. In Levine's study, which focused on microscopic examination of the heart in 31 cases, six were perfectly normal, and 13 with inflammatory infiltrates showed intact heart muscle fibers (18). Twelve cases showed injury to cardiac myocytes, including several with frank focal necrosis that was rarely severe. No measurements of cardiac dynamics were available in that era to distinguish whether the basis of pulmonary edema and peripheral edema was congestive heart failure or noncardiogenic, owing to endothelial injury–based increased vascular permeability in the lungs. Levine concluded on the basis of autopsies of scrub typhus victims who died substantially later that the myocardial changes "eventually disappear, leaving no trace of previous damage" (18, p. 327). We did not observe necrosis of any cardiac myocytes that were infected with *O. tsutsugamushi.*

The demonstration of endothelial cells as the major target cells of *O. tsutsugamushi* in scrub typhus raises the issue of the appropriateness of animal model systems in which endothelial cells have not been reported to be infected. Mice infected with *O. tsutsugamushi* have been used extensively, particularly in studies of anti-*Orientia* immune mechanisms (20–26). Careful immunofluorescent studies of infected mice revealed that the route of inoculation determined the distribution of the organisms (27, 28). Intraperitoneal inoculation resulted in infection predominantly of the peritoneal lining and exudate. Intracerebral inoculation caused infection localized in the leptomeninges. In contrast, intramuscular or subcutaneous inoculation produced disseminated infection involving "Kupffer cells, macrophages, and other forms of mesothelial connective tissue" in the liver, spleen, lungs, kidneys, lymph nodes, thymus, and adrenal glands (27, p. 777). Even considering the limitations of determining the identity of cell types by fluorescence microscopy, one must conclude that there are serious reservations regarding the appropriateness of these mouse models in elucidating the mechanisms of immunity or pathogenesis of human infection with *O. tsutsugamushi.*

Similar questions pertain to cell culture models of *O. tsutsugamushi* infection. Geng and Jerrells (23) demonstrated that recombinant tumor necrosis factor-α inhibited intracellular growth of *O. tsutsugamushi* (Karp strain) in a mouse embryo cell line and in macrophages derived from peritoneal exudate or bone marrow. However, gamma interferon inhibited the growth of *O. tsutsugamushi* only in the murine macrophages and not in the mouse embryo cell line. The cell type was critical in the outcome, in other words, in growth or inhibition of the organisms. Our present study emphasizes that understanding intracellular anti-*Orientia* mechanisms must require investigations in endothelial cells as well as macrophages, including studies in human cells.

Previous studies in our laboratory have demonstrated that IHC is an effective method for detecting *R. prowazekii, R. typhi, R. conorii, R. akari,* and *R. rickettsii* in formalin-fixed paraffin-embedded tissues (29–32). In the present study, the reliable demonstration of *O. tsutsugamushi* by IHC with an anti-*Orientia* antibody confirmed this method as an improved diagnostic tool, as shown by the detection of these organisms in 35- to 57-year-old samples.

It is remarkable that the rabbit polyclonal antibody detected both Karp and Gilliam strains in infected mice as well as the unknown strains of our three human cases. An important antigen for the detection of the spotted fever and typhus groups of *Rickettsia* is the respective group-specific lipopolysaccharides. 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 (8, 33). Indeed it is possible that this particular polyclonal antibody to *O. tsutsugamushi* might not detect all of the multitude of genetically divergent strains. However, within a particular geographic locus where a limited number of strains are in circulation, antibodies could be produced for diagnostic use in identification of *O. tsutsugamushi* in cutaneous biopsies of rash or eschar. Because only a fraction of patients manifest these lesions, immunohistochemical diagnosis cannot be achieved in all cases by this approach. However, in critically ill patients who are admitted late in the course of illness, it would be highly useful to establish the precise diagnosis in as timely a manner as possible to ensure that appropriate treatment is administered, inappropriate treatment is avoided, and attention is alerted for the well-established manifestations and complications of scrub typhus. Evaluation of IHC for diag-

nostic identification of *O. tsutsugamushi* would be particularly important in areas of the world where scrub typhus is endemic.

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