Rickettsia japonica sp. nov., the Etiological Agent of Spotted Fever Group Rickettsiosis in Japan

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We propose the name Rickettsia japonica sp. nov. (with type strain YH [= ATCC VR-1363]) for a serologically specific species of spotted fever group rickettsiae that are pathogenic for humans (J. Infect. Dis. 159:1122–1126, 1989; J. Clin. Microbiol. 28:1177–1180, 1990). The biologic and genomic characteristics of the organism (G+C content, 31.2 ± 0.7 mol%) are essentially the same as those of other pathogenic spotted fever group rickettsiae, although the R. japonica isolates cause a persistent infection in Vero cells for many subcultures.

In this paper we formally describe Rickettsia japonica (23, 25), which has been identified as the causative agent of a human disease. The first isolate, strain YH (T = type strain), was isolated in 1985 from the blood of a patient with febrile exanthematous illness in Japan by using a tissue culture technique (20). Five strains of the causative agent, including strain YH, have been isolated from patients that were serodiagnosed as having a spotted fever group (SFG) rickettsiosis (22).

Justification for a new species. The justification for establishing a new species for this organism is based on the current standard method for comparing rickettsial taxonomic types by serologic analysis (23). Experiments involving reciprocal cross-reactions of mouse polyclonal antibodies to strains of the new species and other species of SFG rickettsiae produced by the standard method were carried out to calculate the specificity differences. By using this approach we demonstrated that all five strains belong to a single species that is distinct from all of the previously described SFG rickettsiae that are known to be pathogenic for humans (23). In addition, none of the isolates reacted with mouse monoclonal antibodies that are species specific for other pathogenic SFG rickettsiae (23). Furthermore, the results of Western immunoblotting revealed different electrophoretic mobilities and antigenic reactivities for the major immunodominant high-molecular-weight surface polypeptides of the Japanese isolates and standard pathogenic SFG rickettsial strains (23). Species-specific monoclonal antibodies to R. japonica reacted only with strains of R. japonica, supporting the conclusion that R. japonica is a new species of SFG rickettsiae (25).

Description of Rickettsia japonica sp. nov. Rickettsia japonica (ja. po’ ni. ca. N.L. adj. japonica, pertaining to Japan, the country in which the organism was first isolated). In smears from cell cultures, the intracellular organisms are visualized by Gimenez staining or by indirect immunofluorescence with immune sera (20). Gram-negative rods that are 0.4 to 0.5 by 0.8 to 1.5 μm. The organisms possess an outer slime layer and a trilaminar cell wall with thin outer and thick inner leaflets (24). Obligate intracellular parasite. The organisms carry species-specific epitopes on the surface (25). The organisms also possess an SFG-common antigen that is detectable by monoclonal antibody 3Y8-B3 (to R. japonica YH1) that reacts with all of the strains of SFG rickettsiae tested but not with Rickettsia typhi Wilmington (= ATCC VR144). Polyclonal antibodies produced in mice against R. japonica react with R. typhi only at low titers. On the other hand, sera from some patients infected with R. japonica react with R. typhi at high dilutions (22).

Growth of R. japonica occurs in Vero cells without cytopathic effects, and a carrier state that persists for 20 subcultures of infected cells develops. Carrier Vero cells divide until a monolayer is formed after subculturing in minimal essential medium containing 10% fetal calf serum and no antibiotics in a 5% CO2-air incubator at 34°C. After cocultivation with uninfected Vero cells, all cells enter the carrier state. The organisms in carrier cells are viable after storage at −80°C or in liquid nitrogen when the carrier cells are stored in minimal essential medium containing 5% dimethyl sulfoxide, 20% fetal calf serum, and no antibiotics. R. japonica differs from other SFG rickettsiae with respect to growth in Vero cells; the strains which we tested have been described previously (23). Rickettsia australis Cutlack, Rickettsia conorii Malish 7, Rickettsia rickettsii R (= ATCC VR891), and Thai tick typhus rickettsia TT-118 produce cytopathic effects 3 to 6 days after inoculation. Although Rickettsia akari Kaplan (ATCC VR148) and Rickettsia sibirica 232 cause few cytopathic effects, the infected cells do not grow continuously after subculturing. The growth properties are the same in Vero C1008 (= ATCC CRL1586) cells. Chicken embryo fibroblast primary culture, BHK 21/13, and L929 cells allow R. japonica to grow with cytopathic effects. Replication in Vero and BHK 21/13 cells occurs primarily in the cytoplasm and rarely in nuclei. Figure 1 shows ultrathin sections of heavily infected Vero C1008 cells, which were examined with a Hitachi model HU-12 electron microscope as described previously (24).

The plaque morphology produced by R. japonica is different from that produced by most other SFG rickettsiae. Infected Vero cells centrifuged at 250 × g for 5 min were diluted in sucrose-phosphate-glutamate buffer (1) and plated onto Vero cell monolayers and then overlaid with Leibovitz medium L-15 containing 0.5% methylcellulose and 5% fetal calf serum. At intervals during incubation at 34°C in an air incubator, the monolayers were simultaneously fixed and stained with a 10% formalin solution containing 0.5% crystal violet and washed with water to identify plaques (infectious centers). After 9 to 11 days of incubation, R. japonica formed plaques with a targetlike appearance (a dye-stained

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targetlike plaques; the one exception was a clear-plaque variant that was isolated from a guinea pig which was inoculated with 10⁶ carrier cells. Specific-pathogen-free strain C3H/HeSlc mice and conventional strain ddY mice (males that were 8 to 12 weeks old) developed inapparent infections after intraperitoneal inoculation; C3H/He mouse strains have been reported to be susceptible to R. conorii (3). The mice produced antibodies (titors, 1:20 to 1:160) on day 7, and the titers increased to 1:320 to 1:2,560 on day 21 after inoculation with 10⁴ to 10⁵ carrier cells. Chicken embryos died 5 to 7 days after yolk sac inoculation with organisms propagated in chicken embryo fibroblasts (10⁷ infected cells) when they were incubated at 33°C; the maximum yield occurred on day 2 after the death of the chicken embryos.

R. japonica exhibited no hemolytic activity when it was assayed with 0.26 mg of protein by using the method of Snyder et al. (14); the organisms which we used were propagated in BHK 21/13 cells and banded at a density of 1.080 g/cm³ in a Percoll gradient. A control reaction mixture containing the same quantity of R. typhi protein resulted in hemolysis, giving an A₄₅₀ of 0.240.

The buoyant density of R. japonica DNA was estimated to be 1.692 g/cm³. Rickettsial DNA extracted from Percoll density gradient-purified organisms by using the standard phenol-chloroform method (12) was centrifuged with CsCl in 10 mM Tris HCl (pH 8.0) at 290,000 × g for 7 h at 20°C in a type RP55VF2 vertical rotor in a Hitachi model CP56G Himac preparative ultracentrifuge, resulting in a single peak of DNA in a CsCl gradient. The CsCl density of the peak fraction, obtained from the refractory index calculations of Marmur and Doty (9), was determined on the basis of a value of 1.710 g/cm³ for the density of the reference DNA from Escherichia coli B (13). Both strain YH¹ and clear-plaque variant DNAs banded at the same density. The thermal denaturation temperature (Tₘ) of DNA from R. japonica was 82.1 ± 0.3°C (n = 5). A Hitachi model U-3210 spectrophotometer equipped with a thermoelectric cell holder connected to a model SPR-10 temperature controller and a digital thermometer was used to measure the Tₘ as described by Marmur and Doty (9). In this study we used clear-plaque variant DNA. As Table 1 shows, reference R. rickettsii R DNA, which was extracted and purified as described above, had a Tₘ identical to the value reported previously (17). An E. coli B standard DNA also had the Tₘ reported previously (9).

The guanine-plus-cytosine (G+C) content of R. japonica DNA calculated from the Tₘ by using the previously de-
scribed formula (9) was $31.2 \pm 0.7$ mol%. This value is identical to the values obtained for other SFG rickettsiae (17). The G+C value calculated from the estimated buoyant density by using the equation described by Schildkraut et al. (13) was 32.6 mol%.

**Description of the illness.** The illness caused by *R. japonica* is found mainly in the southwestern part of Japan (4, 6–8, 15, 16, 18, 19, 21, 26); a few cases have been reported in the west-northwest portion of Honshu (10). *R. japonica* causes an illness (Oriental spotted fever) that is similar to boutonneuse fever. A similar agent has been isolated in another laboratory (11) and remains to be identified as *R. japonica*. The vector has not been determined. The full geographic distribution of the organisms remains to be established.

**Type strain.** Type strain YH (= ATCC VR-1363) of *R. japonica* is the first strain of *R. japonica* that was isolated and has been distributed to the National Institute of Health, Tokyo, Japan, and to prefectural public health laboratories in Japan. All of the strains have the same properties.

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