JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1987, p. 628–632
0095-1137/87/040628-05$02.00/0

Vol. 25, No. 4

Isolation of a Spotted Fever Group Rickettsia from a Patient and Related Ecologic Investigations in Xinjiang Uygur Autonomous Region of China

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Received 10 October 1986/Accepted 30 December 1986

Investigation of patients, healthy persons, and ticks in Jinghe County, Xinjiang Uygur Autonomous Region, People's Republic of China, for evidence of spotted fever group (SFG) rickettsiosis demonstrated strong evidence for a high prevalence of pathogenic SFG rickettsiae. Antibodies to SFG rickettsiae were detected in 62.5% of healthy subjects tested by enzyme-linked immunosorbent assay and 20% tested by complement fixation test. Two febrile patients were documented as having acute spotted fever rickettsiosis by complement fixation seroconversion. One, an 11-year-old Kazakh boy with eschar and regional lymphadenopathy, had an SFG rickettsia (An strain) isolated from his blood. A hemolymph test revealed that 20% of ticks contained rickettsiae. Two strains of SFG rickettsiae were isolated from male and female *Dermacentor nuttalli* ticks. The human SFG rickettsial isolate is the first to be obtained in the People's Republic of China.

A spotted fever group (SFG) rickettsia (Jinghe-74 strain) was isolated in 1974 from *Dermacentor nuttalli* ticks in Jinghe County, Xinjiang Uygur Autonomous Region, People's Republic of China (12). A serosurvey in this area in 1977 demonstrated antibodies to SFG rickettsiae in 71% of a group comprising both healthy persons and suspected patients examined by the complement fixation (CF) test (1). In this report we present the first isolation of SFG rickettsia from a patient in China and clinical and ecologic data supporting the existence of a prevalent tick-borne SFG rickettsiosis in northwestern China.

MATERIALS AND METHODS

Description of the location studied. Kuleti, two smaller villages (Husimutu and Walanggezi), and a forestry center have a population of about 400. They are located 50 km southeast of the Jinghe county seat, latitude 44°31′ north, longitude 88°04′ east, in a mountainous area 1,750 to 2,000 m above sea level. There is more rainfall (320 to 480 mm, mean annual precipitation) mainly between April and July than in the nearby plains. The mean temperatures in 1978 (1) were 6.6°C in April, 8.9°C in May, 10.8°C in June, 16.2°C in July, and 14.2°C in August. The land comprises a forest-edge grassland of 12 km^2.

Sera. Sera were collected from 100 healthy persons ranging in age from 10 to 40 years. Sera were obtained from 19 febrile patients in the acute phase of illness primarily in early May 1984. Convalescence sera were collected from five of these patients 1 month later in June. Sera were transported on ice to the laboratory where they were stored at −30°C until tested.

Serology. The sera were assayed for antibodies to the SFG rickettsial isolate from ticks in 1974 (Jinghe-74) by the CF test and by enzyme-linked immunosorbent assay (26). CF antigen was prepared by cultivation of rickettsiae in yolk sacs of embryonated hen eggs, extraction with ether, and extensive washing. CF antigen was used at a dilution of 1:16.

The enzyme-linked immunosorbent assay was performed in 40-well polystyrene microtiter plates which were coated for 16 h at 4°C with 100 μl of crude antigen consisting of the Jinghe-74 strain of SFG rickettsia cultivated in yolk sacs of embryonated chicken eggs diluted 1:100 in 0.06 M sodium bicarbonate buffer, pH 9.6. Plates were then washed three times with phosphate-buffered saline (0.01 M sodium potassium phosphate, 0.14 M NaCl; PBS) supplemented with 0.05% Tween 20, pH 7.2 (TPBS). Human sera, 100 μl per well, diluted 1:50 in TPBS, were added to each well, incubated at 37°C for 1 h, and washed three times with TPBS. Then, 100 μl of peroxidase-labeled staphylococcal protein A (Department of Immunodiagnostic Products, Institute of Epidemiology and Microbiology, Beijing), diluted 1:50 in TPBS, was added to each well, incubated at 37°C for 1 h, and washed three times with TPBS. *ortho*-Phenylenediamine diluted 1:100 in distilled water was mixed with 100 μl of 3% aqueous H_2O_2, and 100 μl of the mixture was added to each well. The chromogenic reaction developed for 30 min in the dark, and then the reaction was stopped with 50 μl of 2 M H_2SO_4 per well. The optical density was determined at 490 nm. Normal serum and bovine serum albumin controls yielded an optical density of less than 0.1, the threshold for a positive result.

Hemolymph test. Approximately 500 ticks (200 males, 300 females) were collected from *Achnatherum splendens* grass by dragging of cotton flags over grass where ticks had climbed to the top in wait for a passing host. A drop of hemolymph was collected from an amputated leg of each of 40 ticks (4), placed onto a clean glass slide, dried, stained by the Gimenez method (11), and examined microscopically. The remaining ticks were used for isolation of rickettsiae.

Isolation of rickettsiae. Blood samples from each of three patients suspected of having SFG rickettsiosis were inoculated intraperitoneally in 2-ml volumes into pairs of adult male guinea pigs weighing 400 g. Guinea pigs were observed

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daily for signs such as scrotal swelling, and their temperatures were measured twice each day for 3 to 4 weeks. On day 2 of fever, one animal of each pair was sacrificed with aseptic collection of spleen for immediate intraperitoneal passage into two male and two female guinea pigs. Smears of tunica vaginalis were stained by the Gimenez technique (11). Guinea pig passages were continued until the return from the remote field location to facilities where lyophilization of samples was performed. Guinea pig sera were collected at intervals and tested by CF for antibodies to Jinghe-74 strain of SFG rickettsia.

Separated groups of male and female *D. nuttalli* ticks were allowed to feed upon pairs of guinea pigs which were placed into two buckets with the ticks. Guinea pigs were then observed for scrotal swelling, eschars, and fever, and spleen passages were performed as described above.

Lyophilized guinea pig spleen was reconstituted upon the return to Beijing, where yolk sacs of embryonated hen eggs were inoculated with this material (26). Yolk sacs of eggs in which the embryo died more than 48 h after inoculation were incubated a further 48 h after embryo death and harvested. Smears were examined by Gimenez stain and indirect-fluorescent-antibody stain with a rabbit anti-SFG rickettsial serum and goat anti-rabbit serum conjugated to fluorescein isothiocyanate (26). Preliminary identification of rickettsial isolates was performed on smears of each rickettsial isolate cultivated in a yolk sac of embryonated chicken eggs. Rickettsial smears were applied to clean glass slides and fixed for 7 min in acetone before indirect immunofluorescence staining.

Identification of isolates as SFG rickettsiae. Indirect-immunofluorescent-antibody tests were performed on acetone-fixed microdots of the following cell culture-cultivated antigens: *Rickettsia rickettsii* (Sheila Smith strain), *Rickettsia sibirica* (strain 232), An strain, XJFT-84, and XJMT-84 in Vero cells, and *Rickettsia conorii* (Malish 7 strain) in primary chicken embryo cells. Convalescent-phase antisera from guinea pigs inoculated intraperitoneally with the same strains of rickettsiae cultivated in yolk sacs of embryonated chicken eggs, except for An strain from primary chicken embryo cell cultures, were used as antibodies, and normal guinea pig serum was used as a control. Sera diluted 1:256 in PBS were incubated on microdots in a moist chamber at room temperature for 30 min and washed for 5 min in PBS. The slides were then allowed to react with rabbit anti-guinea pig immunoglobulin-fluorescein isothiocyanate (Accurate Chemical & Scientific Corp., Westbury, N.Y.) diluted 1:40 in PBS, washed again for 5 min in PBS, and mounted in 90% glycerol–10% PBS. Slides were examined on a Leitz Laborlux 12 UV microscope (Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, N.J.) with epi-illumination and barrier and exciter filters for fluorescein.

Histopathology of infected guinea pigs. Organs from sacrificed guinea pigs were removed and fixed in 4% formaldehyde or 2.5% glutaraldehyde, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

RESULTS

There was a high prevalence of antibodies to SFG rickettsiae among healthy subjects tested with 40 (62.5%) of 64 positive by enzyme-linked immunosorbent assay and 19 (20%) of 96 positive by CF. Of five sera from convalescent patients, two contained CF antibodies to SFG rickettsiae at titers of 1:32 and 1:64, as compared with negative acute-phase sera.

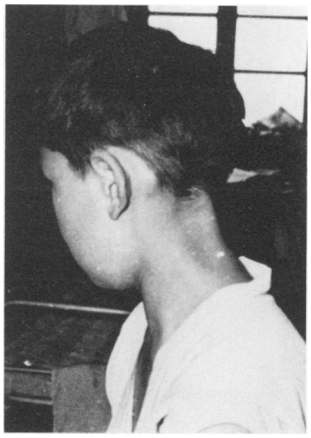

FIG. 1. An 11-year-old Kazakh boy from whom An strain SFG rickettsia was isolated had left posterior cervical lymphadenopathy related to an eschar of the scalp (not shown).

An 11-year-old Kazakh boy was bitten on the scalp by a tick on 3 May 1984. He subsequently developed an eschar at the site of the tick bite and regional lymphadenopathy (Fig. 1), and SFG rickettsiae were isolated by inoculation of his blood into guinea pigs (see below). His illness was mild to moderate with elevated concentrations of alanine aminotransferase in serum of 100 U/liter on 22 May and 50 U/liter on 1 June (normal value, less than 40 U/liter), aspartate aminotransferase concentrations of 48 and 32 U/liter (normal range, 8 to 40 U/liter) on the same days, respectively, and normal levels in serum of amylase, fibrinogen, and nonprotein nitrogen. The titer of CF antibodies to SFG rickettsiae rose acutely from less than 1:8 to 1:64 on 1 June.

Of 40 ticks examined by the hemolymph test, 8 (20%) contained identifiable rickettsiae.

Three strains of SFG rickettsiae were isolated, one each from a patient (An strain), male *D. nuttalli* ticks (XJMT-84), and female *D. nuttalli* ticks (XJFT-84). Of the six guinea pigs inoculated with blood from patients, only one developed a fever above 39.6°C on days 10 to 13 after inoculation. Spleen collected from this animal and passaged into four more guinea pigs produced fever in all of them and scrotal reaction in both males. One animal, which became febrile on day 4 with a temperature greater than 40°C on days 4 to 8, was sacrificed on day 8. The spleen was harvested, lyophilized, and passaged into yolk sacs of embryonated chicken eggs. One female guinea pig had anti-SFG rickettsial CF antibody titers on days 0, 14, and 21 that were negative, 1:16, and 1:128, respectively. Likewise, one male guinea pig in the third passage had anti-SFG rickettsial CF titers on days 0, 14, and 21 that were negative, 1:16, and 1:128, respectively. Once established in guinea pigs, An strain had an incubation period of 3 to 5 days, followed by fever (40 to 41°C) for 4 to 6 days.

All four guinea pigs fed upon by ticks developed fever after 2 to 5 days and at the time of sacrifice on day 4 to 5 of fever had approximately 10 eschars per animal. Rickettsiae were observed in Gimenez-stained tunica vaginalis smears. Three subsequent serial passages produced fever in all guinea pigs inoculated with spleens collected from the previous passage. Scrotal congestion and edema were observed in some animals of the second and third generations. Spleens

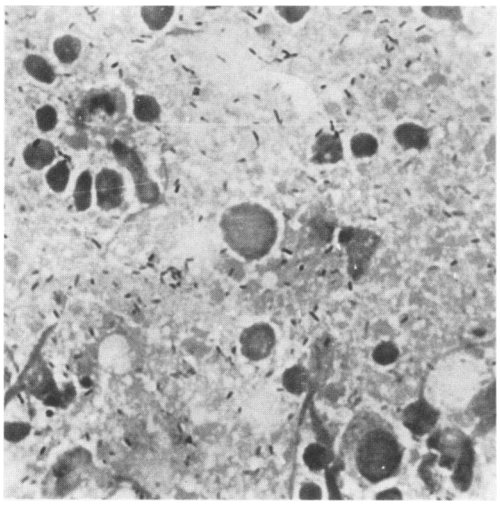

FIG. 2. Smear of sixth yolk sac passage containing An strain SFG rickettsiae isolated from a patient in Xinjiang. Gimenez stain. Magnification, ×1,000.

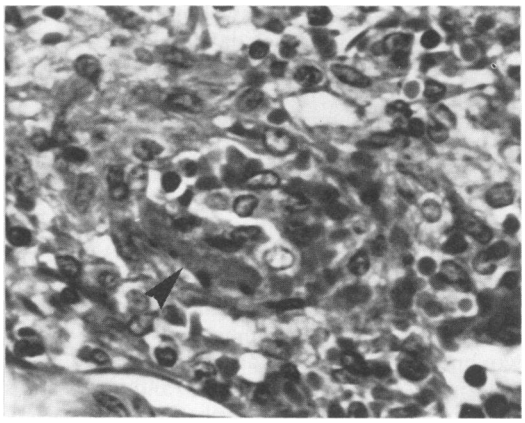

FIG. 4. Deep dermis underlying an eschar at the site of tick bite in the guinea pig from which XJFT-84 strain SFG rickettsia was isolated. A blood vessel has endothelial injury, a nonocclusive thrombus (arrow), and perivascular hemorrhage and mononuclear leukocytes. Hematoxylin and eosin stain. Magnification, ×785.

from the fourth generation were lyophilized, transported to Beijing, and inoculated into yolk sacs of embryonated ckicken eggs. SFG rickettsiae were identified by indirect immunofluorescence in the first yolk sac passage from the lines of passage from a guinea pig fed upon by male ticks and from a guinea pig fed upon by female ticks. Subsequent serial yolk sac passages established good growth of all three strains of rickettsiae detectable by both Gimenez stain (Fig. 2 and 3) and indirect immunofluorescence with rabbit anti-Jinghe-74 SFG rickettsial serum and goat anti-rabbit immunoglobulin-fluorescein isothiocyanate conjugate.

All of the guinea pig antisera to the prototype SFG rickettsial species (*R. rickettsii*, *R. conorii*, and *R. sibirica*) and to the Xinjiang strains (An, XJFT-84, and XJMT-84) cross-reacted with all of the same set of rickettsial antigens by microimmunofluorescence at a titer of 1:256. None of the rickettsial antigens reacted with normal guinea pig sera. These results confirm the identification of these organisms as members of the SFG.

Histopathology of infected guinea pigs. The cutaneous site of attachment of one of the female ticks had epidermal necrosis with focal ulceration, crust formation, and intense infiltration of the superficial dermis with a mixed leukocyte population including many polymorphonuclear leukocytes. The deep dermis and superficial subcutaneous tissue contained small arteries and veins with swollen endothelium, focal nonocclusive thrombosis, focal hemorrhage, and marked intramural and perivascular infiltration by mononuclear cells (Fig. 4).

The organs from one of the guinea pigs infected with XJFT-84 strain SFG rickettsia showed multifocal hepatic

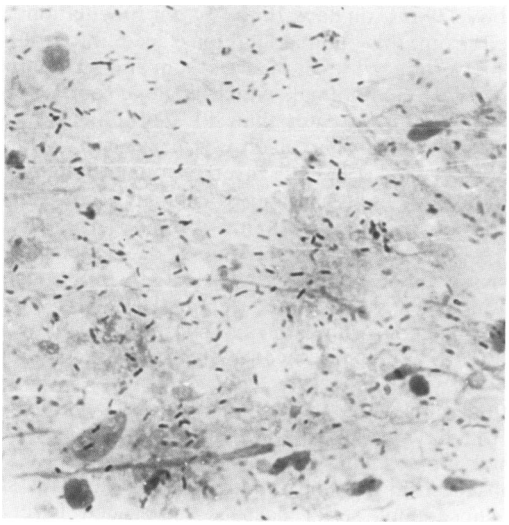

FIG. 3. Smear of fourth yolk sac passage containing XJFT-84 strain SFG rickettsiae isolated from female *D. nuttalli* in Xinjiang. Gimenez stain. Magnification, ×1,000.

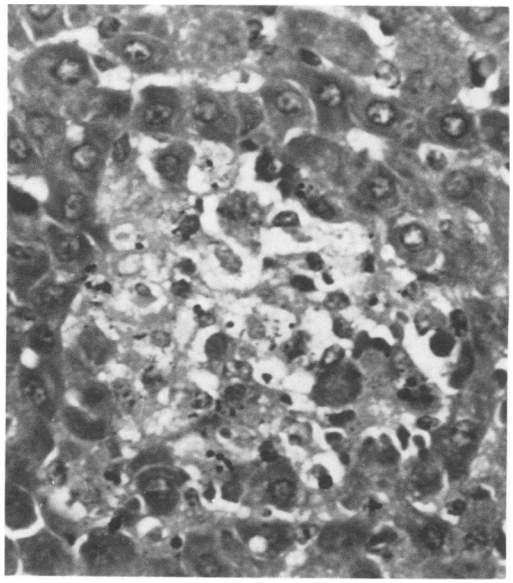

FIG. 5. Focal hepatic necrosis in a guinea pig infected with XJFT-84 SFG rickettsia. Hematoxylin and eosin stain. Magnification, ×785.

necrosis with mild mononuclear-cell response (Fig. 5) and evidence for multifocal vascular injury.

DISCUSSION

Our reported case of a Kazakh boy with SFG rickettsiosis is the first to be documented by isolation of SFG rickettsiae in the People's Republic of China (9). The case is further confirmed by CF test seroconversion.

R. sibirica is the species identified as the etiologic agent of North Asian tick-borne typhus. However, in general, any SFG rickettsia or rickettsial illness within northeastern Asia is considered to be *R. sibirica* on a geographic basis. Accepted rickettsiologic methods for distinguishing species of SFG rickettsiae are the mouse toxicity neutralization test and microimmunofluorescence (3, 21). Few isolates of SFG rickettsiae from eastern Asia have been examined by these empiric assays, for which the molecular bases are unknown (3, 21, 22). Work is currently in progress in our laboratories to analyze the antigens, polypeptides, and DNAs of these rickettsiae along with prototype strains by microimmunofluorescence, Western immunoblotting, monoclonal antibodies, and restriction endonuclease methods.

SFG rickettsiae have been documented as highly endemic in numerous locations in northern China. Antibodies to SFG rickettsiae have been demonstrated in healthy persons in other studies in Inner Mongolia and Heilongjiang Province (2.5 to 17.3% by CF test and 21% by indirect-fluorescent-antibody test) (10, 15). SFG rickettsiae have also been isolated from six species of ticks (*Haemaphysalis concinna*, *Dermacentor nuttalli*, *D. japonica*, *D. marginatus*, *D. silvarum*, and *D. niveus*) in the same regions (8, 10, 13, 14, 16, 27), from a rodent, *Microtus fortis*, in Heilongjiang (25), and subsequently also by our group of investigators from a patient in Inner Mongolia in 1985 (10).

The demonstration of rickettsiae in 20% of ticks in this area suggests that a high rickettsial infection rate in ticks accounts for the high prevalence of antibodies (62.5% by enzyme-linked immunosorbent assay) in the human population. The lower seroprevalence rate detected by CF test was expected, since this assay reverts to negative in a higher proportion of patients after SFG rickettsiosis. Moreover, we observed that the incidence of spotted fever in this location increased sharply shortly after the appearance of *D. nuttalli* in the spring with peak occurrence in late April to early May, particularly in school-age children. Indeed, two strains of SFG rickettsiae that are pathogenic for guinea pigs were isolated from ticks in this study. It seems unlikely that SFG rickettsiae causing rickettsemia, fever, eschars, and hepatic and vascular lesions in guinea pigs analogous to human lesions previously reported for *R. sibirica* and *R. conorii* would be nonpathogenic (17, 18, 23, 24). However, it is conceivable that nonpathogenic rickettsiae analogous to nonpathogenic SFG rickettsiae in ticks of North America and Europe may also exist in Asia (2, 5–7, 19, 20). There are at least two species of SFG rickettsiae that have been isolated from ticks in Asia for which the pathogenicity in humans is not known (22). This is the general situation regarding rickettsiae obtained from arthropod sources and serves to reemphasize the importance of obtaining human isolates for analysis in addition to rickettsiae from ecologic and epidemiologic investigations.

ACKNOWLEDGMENTS

We thank Lorraine Zeiler for assistance in the preparation of the manuscript.

This work was supported in part by Public Health Service grant AI-22224 from the National Institute of Allergy and Infectious Diseases.

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