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Serodiagnosis of Tsutsugamushi Fever (Scrub Typhus) by the Indirect Immunoperoxidase Technique

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The indirect immunoperoxidase technique was assessed for the serodiagnosis of tsutsugamushi fever (scrub typhus). The antigens were peritoneal smears prepared from mice infected intraperitoneally with the Karp, Kato, and Gilliam strains of *Rickettsia tsutsugamushi*. Treatment of the mice with cyclophosphamide apparently increased the number of the rickettsiae, and it minimized the exudate that interfered with the specific staining. The rickettsiae were seen as clusters in the juxtanuclear region of the mesothelial cells and also as free particles outside of the cells. By the indirect immunoperoxidase technique, the sera from all of the patients (49 samples from 30 patients) were positive for the *R. tsutsugamushi* antibody. The antibody titers (immunoglobulin G [IgG] and IgM) determined by the indirect immunoperoxidase technique correlated with those determined by the indirect immunofluorescence technique. Thus, the indirect immunoperoxidase technique was useful for quantifying both IgG and IgM antibodies to the rickettsia.

Serodiagnosis of tsutsugamushi fever (scrub typhus) with *Rickettsia tsutsugamushi* antigens has been accomplished by the complement fixation test (6, 9), the indirect immunofluorescence (IIF) technique (1, 4), and the enzyme-linked immunosorbent assay (ELISA) (2, 3). The IIF technique has been used effectively and is preferred for the laboratory diagnosis of tsutsugamushi fever (8). In general, the IIF technique gives a broader reaction with strains of *R. tsutsugamushi* than the complement fixation test does (8), although a cross-reacting complement-fixing antigen has been reported (6, 9). In addition, the IIF technique can quantify either immunoglobulin G (IgG) or IgM antibodies independently. The indirect immunoperoxidase (IIP) technique, in which fluorochrome is replaced by peroxidase, has several advantages over the IIF technique and has been widely applied in diagnostic virology (7). Accordingly, we investigated the IIP technique as an alternative to the IIF technique for detecting and measuring antibodies to *R. tsutsugamushi*. This paper is concerned with the procedure and the evaluation of the IIP technique for serodiagnosis of tsutsugamushi fever.

MATERIALS AND METHODS

Rickettsiae. The Karp, Kato, and Gilliam strains of *R. tsutsugamushi* were used for the preparation of antigens. The rickettsiae, supplied by A. Kawamura, Jr., University of Tokyo, had been passed in ddY mice by the intraperitoneal route.

Preparation of antigen smears. A 10% homogenate of spleens of mice infected with each strain of *R. tsutsugamushi* was prepared in phosphate-glutamate-sucrose solution (0.0038 M KH_2PO_4, 0.0072 M K_2HPO_4, 0.0049 M L-glutaminic acid, 0.218 M sucrose [pH 7.0]). A 0.3-ml amount of rickettsial suspension was inoculated intraperitoneally into mice. At 1 and 2 days later, 0.2 mg of cyclophosphamide (Shionogi and Co. Ltd., Osaka, Japan) per g (mouse weight) was injected into the mice intraperitoneally (11). Peritoneal smears were made from the infected mice 10 days after inoculation. The smears were air dried, fixed in cold acetone for 10 min, and stored at 4°C and at −20°C for IIP and IIF staining, respectively, until use. When stored as above, no deterioration of the antigenicity of each antigen was observed over a period of 2 to 3 months. Control antigens consisted of smears similarly prepared from uninfected mice treated with cyclophosphamide.

Serum specimens. Thirty-two serum specimens from 16 patients with tsutsugamushi fever were provided by I. Sasakawa, Public Health Laboratory, Niigata prefecture, Japan, and A. Kawamura, Jr. (group 1). Seventeen serum specimens were collected from 14 patients in the Miyazaki and Kagoshima prefectures during the recent endemic of 1980 to 1981 (group 2). Forty serum specimens were obtained from healthy individuals with no history of tsutsugamushi fever (group 3).

Staining procedures. Demarcation lines were marked with nail polish to make 10 or 12 sections on the antigen slides. This made it possible to titrate one serum sample on one antigen slide. The serum specimens were diluted serially (twofold), starting at 1:20 with phosphate-buffered saline. Then, a drop of each dilution was transferred individually to one section (approximately 0.025 ml per section) and allowed to react with the antigen for 30 min for IgG antibody

determinations and 1 h for IgM antibody assays at room temperature (20 to 25°C) in a moist chamber. The antigen slides were washed in phosphate-buffered saline (four changes) and then were allowed to react as above with peroxidase-labeled goat IgG fractions against human immunoglobulins at a 1:75 dilution. These fractions were anti-IgG:F(ab')$_2$, anti-IgG:Fc (gamma chain), and anti-IgM (mu chain) from Cappel Laboratories, Cochranville, Pa. In the blocking tests, unlabeled goat IgG fractions against human IgG (gamma chain) and IgM (mu chain) (Cappel Laboratories) were used before the reaction with the peroxidase-labeled goat IgG fractions against human IgG:Fc and IgM, respectively. Then the slides were washed as before, rinsed in 0.05 M Tris-hydrochloride buffer (pH 7.6) for 5 min, and allowed to react with a substrate solution for 10 min at room temperature in a box to protect the slides from light. This solution consisted of diaminobenzidine (0.05%) and hydrogen peroxide (0.01%) in the Tris-hydrochloride buffer. The stained slides were washed with distilled water (two changes),

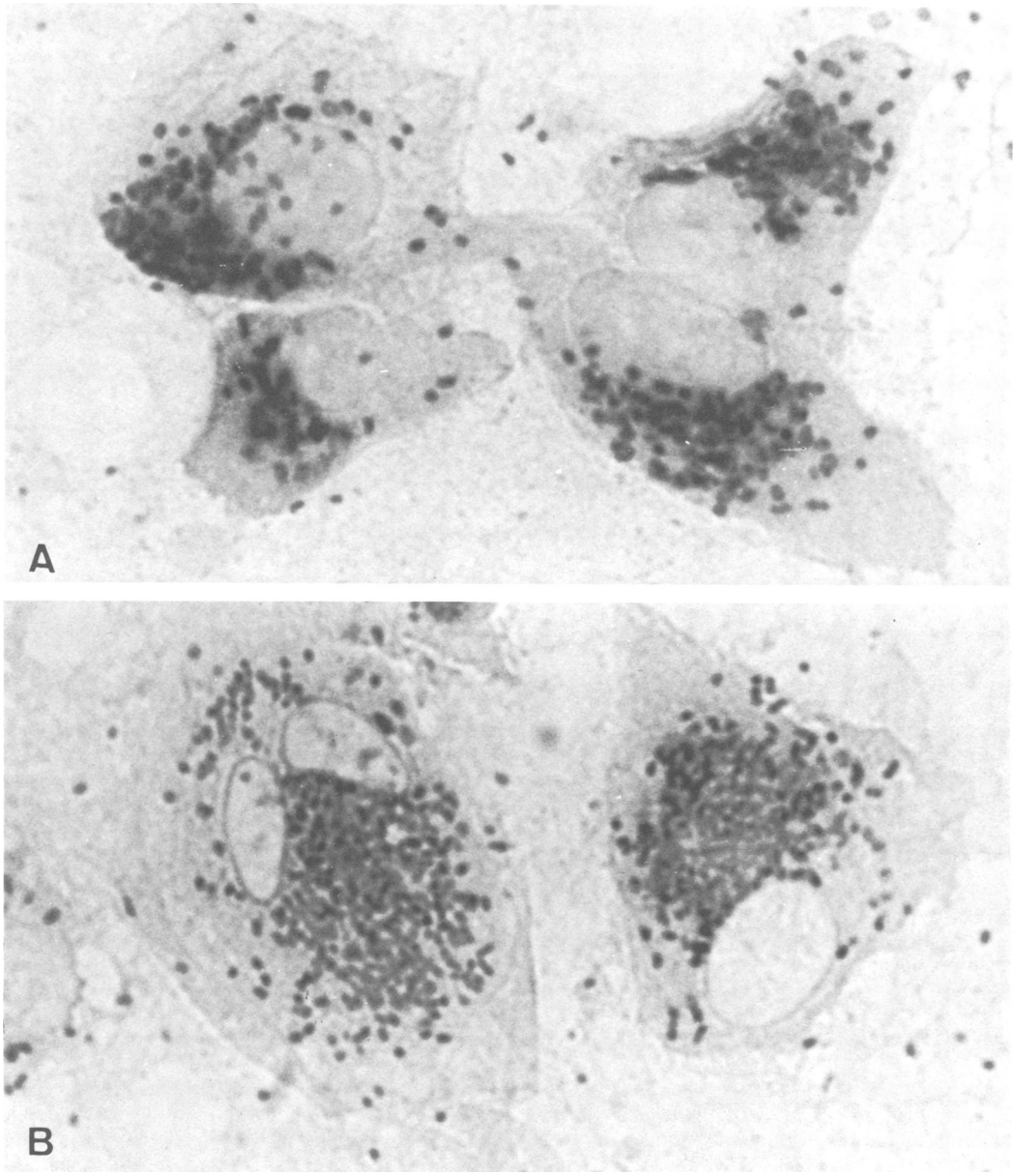

FIG. 1. *R. tsutsugamushi* stained by the IIP technique. Primary antibodies were IgG antibodies of a patient's serum (1:40) in group 1, and secondary antibodies were peroxidase-labeled goat IgG fractions (1:75) against human IgG:F(ab')$_2$ (A) and human IgG:Fc (B), respectively.

TABLE 1. *R. tsutsugamushi* antibodies (IgG) in patients' sera (group 1)

Patient	Days after onset	Rickettsial antigens					
		Karp		Kato		Gilliam	
		IIP	IIF	IIP	IIF	IIP	IIF
1.	7	640[a]	320	ND[b]	ND	ND	ND
	15	320	320	640	640	80	160
	61	640	640	640	1,280	160	320
	83	640	1,280	640	1,280	640	320
	124	320	320	640	640	320	320
2.	11	40	40	ND	ND	ND	ND
	35	640	320	320	320	80	80
	60	1,280	640	640	320	80	40
	80	640	640	320	640	160	160
	122	640	640	640	320	160	320
3.	22	640	160	ND	ND	ND	ND
	64	160	80	320	320	40	20
	104	320	160	160	160	80	80
4.	7	160	40	ND	ND	ND	ND
	23	640	320	640	640	640	320
	64	1,280	320	ND	ND	ND	ND
	391	80	40	80	80	40	40
5.	UK[c]	1,280	1,280	ND	ND	ND	ND
	UK	1,280	640	640	640	160	80
	UK	320	320	1,280	640	160	160
6.	UK	320	160	160	160	320	160
7.	UK	40	40	40	40	40	20
8.	UK	1,280	1,280	1,280	1,280	1,280	1,280
9.	UK	160	80	80	160	40	40
10.	UK	320	320	640	640	160	160
11.	UK	320	320	1,280	1,280	640	320
	UK	1,280	640	1,280	1,280	640	320
12.	UK	320	320	320	320	160	320
13.	UK	640	320	640	640	640	640
14.	UK	20	20	20	20	20	20
15.	UK	640	1,280	ND	ND	ND	ND
16.	UK	320	640	ND	ND	ND	ND

[a] Titers are expressed as the reciprocal of the highest serum dilution that was positive for the indicated strain.
[b] ND, Not determined.
[c] UK, Unknown.

dehydrated through an alcohol series to xylene (two changes), mounted with Diatex (AB Wilh Becker Industrial Paint, Märsta, Sweden), and examined at ×400 under a light microscope.

Another set of antigen slides prepared from the same mice was stained by the IIF technique (5) with fluorescein isothiocyanate-labeled rabbit IgG fractions (diluted 1:40) against human IgG or IgM (Behring Institut, Marburg-Lahn, West Germany). The slides were examined at ×400 with a fluorescence microscope (Nikon model VFD-T) equipped with a mercury lamp (type USH-200D, Usio Electric Inc., Tokyo, Japan), a UV exciting filter (model UV330-380), and a UV exclusion filter (410 W).

RESULTS

Demonstration of *R. tsutsugamushi* by the IIP technique. The most satisfactory antigens were obtained from mice treated with cyclophosphamide after infection. First, the drug treatment apparently increased the number of *R. tsutsugamushi* in the peritoneal cavity, although this was not quantitatively determined. Second, it reduced the peritoneal exudate (cells and fluid). The peritoneal smears of the treated mice consisted mainly of mesothelial cells (lining cells) free of exudate cells carrying Fc receptors or endogenous peroxidase. Consequently, the nonspecific background staining that interfered with the specific staining of the rickettsiae was minimized.

The rickettsiae were seen as brown particles clustered in the juxtanuclear region of the cells. Frequently, the rickettsiae were also observed extracellularly as free particles. The rickettsiae were stained with both anti-IgG:F(ab')$_2$ and anti-IgG:Fc antibodies labeled with peroxidase (Fig. 1A and 1B). Although the staining was usually more intense with the former, there was little difference between the endpoints determined with the two labeled antibodies. Therefore, the former was used as the secondary antibody for detecting IgG antibodies, unless otherwise stat-

TABLE 2. *R. tsutsugamushi* antibodies (IgM and IgG) in patients' sera (group 2)

Patient	Days after onset	IgM antibodies						IgG antibodies					
		Karp		Kato		Gilliam		Karp		Kato		Gilliam	
		IIP	IIF	IIP	IIF	IIP	IIF	IIP	IIF	IIP	IIF	IIP	IIF
1.	11	1,280[a]	1,280	1,280	1,280	640	640	160	320	160	320	80	160
	19	640	1,280	640	640	320	320	320	ND[b]	320	ND	80	ND
2.	8	2,560	2,560	1,280	640	5,120	5,120	160	320	160	160	320	320
	15	1,280	1,280	1,280	640	2,560	2,560	160	ND	160	ND	320	ND
3.	18	2,560	2,560	1,280	1,280	10,240	5,120	2,560	2,560	1,280	2,560	2,560	1,280
	25	1,280	1,280	640	1,280	5,120	5,120	2,560	ND	1,280	ND	2,560	ND
4.	9	640	640	640	640	640	640	640	320	640	320	640	320
5.	31	320	320	160	160	80	80	640	320	320	160	160	80
6.	10	160	160	80	80	40	80	20	40	20	40	<20	<20
7.	21	160	160	320	320	640	640	160	160	80	160	320	320
8.	UK[c]	1,280	640	640	320	2,560	2,560	160	ND	160	ND	320	ND
9.	UK	20	40	20	40	320	640	640	ND	160	ND	640	ND
10.	UK	40	40	80	80	1,280	2,560	320	160	80	160	320	80
11.	UK	320	320	160	160	640	640	80	ND	40	ND	160	ND
12.	UK	640	640	320	320	160	320	1,280	ND	640	ND	160	ND
13.	UK	160	160	80	40	80	80	80	ND	40	ND	20	ND
14.	UK	640	320	640	320	1,280	1,280	40	ND	40	ND	160	ND

[a] Titers are expressed as the reciprocal of the highest serum dilution that was positive for the indicated strain.
[b] ND, Not determined.
[c] UK, Unknown.

ed. The rickettsial particles were similarly stained with anti-IgM (mu chain) antibodies labeled with peroxidase. However, the intracellular rickettsiae were stained faintly in some preparations, whereas the extracellular rickettsiae were stained deeply under the same condition. This was most likely due to poor penetration of IgM antibodies into the cells. Accordingly, for quantification of IgG and IgM antibodies, clusters of the rickettsiae in the cytoplasma and the extracellular rickettsiae were used as the target antigens, respectively.

Specificity of the IIP staining. Each strain of *R. tsutsugamushi* was stained only in the combination of a smear from the infected mouse, a patient's serum, peroxidase-labeled anti-human IgG or anti-human IgM goat IgG fraction, and the substrate solution. All of the sera from the patients (groups 1 and 2) contained antibodies to *R. tsutsugamushi* (Tables 1 and 2). None of the control sera (group 3) diluted 1:20 reacted with the rickettsiae (Karp strain). The specific staining of the rickettsiae was blocked by unlabeled anti-IgG or anti-IgM goat IgG fraction when the two were allowed to react before being stained with peroxidase-labeled anti-IgG or anti-IgM goat IgG fraction and the substrate.

Cross-reaction of *R. tsutsugamushi* antibodies. Sera from the tsutsugamushi fever patients were titrated by the IIP and IIF techniques with the Karp, Kato, and Gilliam strains. Sera which showed an antibody titer of 20 or greater were considered positive for the strain. With 24 samples from 14 Niigata patients who were tested by both procedures against all three strains, IgG antibody to one strain cross-reacted with others (Table 1). This was also the case with IgM antibodies detected in 17 samples from 14 Miyazaki-Kagoshima patients. With IgG antibody, however, there was one patient for whom the antibody titer was less than 20 against the Gilliam strain, but was positive for the others (Table 2). Although in some patients serum antibody titers were higher against certain strains, it was not possible to determine the antigenic type of rickettsiae that caused an endemic in the Miyazaki-Kagoshima area from the IgG or IgM titers against the three strains of *R. tsutsugamushi* (Table 2). Both IgG and IgM antibody titers determined by the IIP technique correlated with those determined by the IIF technique (Tables 1 and 2).

DISCUSSION

Attention should be drawn to the fact that tsutsugamushi fever is increasing in Japan. At least 234 patients, including 3 fatal cases, were registered in 1980 (A. Kawamura, Jr., personal communication). It is of great importance that most cases of the disease are neither concentrated in the formerly endemic areas along certain rivers nor restricted to the summertime. It should be also noted that the usefulness of the Weil-Felix reaction had been exaggerated when the test was the only available one for the laboratory diagnosis of the disease. Actually, sera from two of four fatal cases were negative

for the Weil-Felix reaction with the *Proteus* OXK strain (10). Since the disease usually occurs in the countryside, there is an urgent need for a specific test which can be done routinely in any laboratory.

As reported here, the IIP technique is useful for detecting antibodies to *R. tsutsugamushi*. First, the IIP technique shares the advantages over the complement fixation test with the IIF technique in that any rickettsial strain can be used as the antigen, and either IgG or IgM antibodies can be titrated individually. Second, the IIP technique has the following advantages over the IIF technique: (i) it gives permanent preparations for reexamination; (ii) it does not require a fluorescence microscope; (iii) it makes it possible to observe all cells, infected and uninfected; (iv) it is relatively easy to determine antibody endpoints; and (v) the reagent (the labeled antibody) is also applicable for use in electron microscopy and ELISA. However, the fact remains that in the IIP technique readings are still subjective. In this respect the IIP is inferior to the ELISA (2, 3), by which antibodies can be objectively quantified. One limitation common to the IIP, IIF, and ELISA techniques is the unavailability of the rickettsial antigens from commercial sources.

Recently, Suto and Fujimiya titrated both IgG and IgM antibodies by using extracellular *R. tsutsugamushi* grown in L cells and released by freeze thawing (T. Suto, personal communication). By our method, the antigens, including extracellular rickettsiae, can be prepared without any equipment for cell culture. However, the treatment of the infected mice with cyclophosphamide seems to be a prerequisite for preparation of satisfactory antigens.

In conclusion, the IIP technique with *R. tsutsugamushi* antigens prepared by either method is a satisfactory alternative to the IIF, and it will facilitate early serodiagnosis of tsutsugamushi fever.

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LITERATURE CITED

1. **Bozeman, F. M., and B. L. Elisberg.** 1963. Serological diagnosis of scrub typhus by indirect immunofluorescence. Proc. Soc. Exp. Biol. Med. **112**:568–573.
2. **Crum, J. W., S. Hanchalay, and C. Eamsila.** 1980. New paper enzyme-linked immunosorbent technique compared with microimmunofluorescence for detection of human serum antibodies to *Rickettsia tsutsugamushi*. J. Clin. Microbiol. **11**:584–588.
3. **Dasch, G. A., S. Halle, and A. L. Bourgeois.** 1979. Sensitive microplate enzyme-linked immunosorbent assay for detection of antibodies against the scrub typhus rickettsia, *Rickettsia tsutsugamushi*. J. Clin. Microbiol. **9**:38–48.
4. **Iida, T., K. Okubo, and M. Ishimaru.** 1966. Immunofluorescence for seroepidemiological study of tsutsugamushi disease rickettsia. Jpn. J. Exp. Med. **36**:435–447.
5. **Kawamura, A., Jr.** 1977. Staining methods, p. 77–94. *In* A. Kawamura, Jr. (ed.), Fluorescent antibody techniques and their applications, 2nd ed. University Park Press, Baltimore, Md.
6. **Kobayashi, Y., K. Nagai, and N. Tachibana.** 1969. Purification of complement-fixing antigens of *Rickettsia orientalis* by ether extraction. Am. J. Trop. Med. Hyg. **18**:942–952.
7. **Kurstak, E., P. Tijssen, and C. Kurstak.** 1977. Immunoperoxidase technique in diagnostic virology and research: principles and applications, p. 403–448. *In* E. Kurstak and C. Kurstak (ed.), Comparative diagnosis of viral diseases: human and related viruses, vol. IIB. Academic Press, Inc., New York.
8. **Ormsbee, R. A.** 1974. Rickettsiae, p. 923–933. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
9. **Shishido, A., M. Hikita, T. Sato, and S. Kohno.** 1969. Particulate and soluble antigens of *Rickettsia tsutsugamushi* in the complement fixation test. J. Immunol. **103**:480–490.
10. **Suzuki, T., T. Suto, M. Harada, J. Otsuji, M. Ito, H. Sekikawa, K. Kojima, and H. Kaneko.** 1981. Four fatal cases of tsutsugamushi disease (scrub typhus) occurred in Akita and Niigata prefectures. Akita J. Med. **7**:303–313. (In Japanese.)
11. **Tachibana, N., and Y. Kobayashi.** 1975. Effect of cyclophosphamide on the growth of *Rickettsia sennetsu* in experimentally infected mice. Infect. Immun. **12**:625–629.