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Use of Monoclonal Antibodies against *Rickettsia tsutsugamushi* Kawasaki for Serodiagnosis by Enzyme-Linked Immunosorbent Assay

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Monoclonal antibodies (MAbs) against *Rickettsia tsutsugamushi* Kawasaki were prepared. The cross-reactivity tests of the MAbs performed by using antigenically distinct strains of *R. tsutsugamushi* in immunofluorescence and immunoblotting analyses indicated that the Kawasaki strain contains a strain-specific epitope and also contains a common epitope on the 56-kDa polypeptide cross-reactive with the Gilliam strain, group- and subgroup-specific epitopes on the 46-kDa polypeptide, and a subgroup-specific epitope on the 25-kDa polypeptide. By using the strain-specific MAb for serodiagnosis of tsutsugamushi disease (or scrub typhus fever), we have established a method which was designated the inhibition enzyme-linked immunosorbent assay. The principle of the method is to measure the percentage of inhibition of antigen adsorption on a MAb-coated plate by antibody-positive sample sera which were mixed with the antigen suspension. The advantages of this test for practical use are that (i) crude antigen can be used, i.e., purification of the antigen is not required; (ii) the test is more sensitive than immunofluorescence; (iii) the final judgment of plus or minus is clear-cut; and (iv) rickettsial antigenic types in the patients can be distinguished by this test.

Rickettsia tsutsugamushi, the causative agent of tsutsugamushi disease, or scrub typhus fever, is unique among the *Rickettsia* spp. because of the presence of various antigenic variants. The antigenic types of three prototype strains, Gilliam, Karp, and Kato, are well known. Our recent works have demonstrated the presence of other antigenic types such as Shimokoshi, Kawasaki, and Kuroki strains which are distinguishable not only from the prototype strains but also from one another (9, 13, 14). Furthermore, localized distributions of these antigenic types were observed (6, 13). For example, all *R. tsutsugamushi* isolates from patients in Miyazaki prefecture were Kawasaki and Kuroki types, while the majority of isolates from Niigata prefecture are Gilliam and Karp types. These recent findings have been made possible by the progress of methods for antigenic analysis of rickettsiae, especially by the use of various kinds of antirickettsial monoclonal antibodies (MAbs) (6, 8, 14). Although recent serodiagnoses of tsutsugamushi disease have been performed mainly by the methods of immunofluorescence assay (IF) or immunoperoxidase treatment, the development of other simple, convenient, and accurate methods is still being investigated (11).

In the present study, a modified enzyme-linked immunosorbent assay (ELISA) was developed and applied for serodiagnosis of tsutsugamushi disease by using strain-specific MAbs against the Kawasaki strain which we had prepared. The method is as follows. Wells of an ELISA microplate are coated with Kawasaki strain-specific MAb. Then, crude rickettsial antigen (Kawasaki strain antigen) which was mixed with a test serum is adsorbed to the wells, and the percentage of inhibition of the antigen adsorption by the test serum is measured by ELISA using a peroxidase-conjugated Kawasaki strain-specific MAb. We designated this method the inhibition ELISA. In the first part of the present paper, we describe the preparation and characterization of MAbs against the Kawasaki strain, and in the second part we describe the usefulness of the inhibition ELISA for serodiagnosis of rickettsial infection by *R. tsutsugamushi* Kawasaki, especially in the acute phase of infection.

MATERIALS AND METHODS

Rickettsial strains. *R. tsutsugamushi* Gilliam, Karp, Kato, Shimokoshi, Kawasaki, and Kuroki were used. The first three strains are laboratory strains which have been commonly used as prototypes. The last three were isolated from patients in Niigata and Miyazaki prefectures, and partial characterization of their properties was reported previously (9, 13, 14). All of these strains were propagated in cultures of GMK cells, Vero cells (both originated from green monkey kidney), or L cells (mouse fibroblasts). The detailed procedures for cultivation and propagation were the same as those described previously (13, 14).

Preparation of MAbs. The details were essentially the same as those described previously (2). Briefly, a suspension of Kawasaki strain antigen prepared from the infected GMK cell cultures was emulsified in Freund's complete adjuvant, and the emulsion was injected intraperitoneally into BALB/c mice. After 2 months, the mice were boosted once intravenously by injecting the same antigen; on day 3 after the booster injection, the spleen cells were fused with mouse myeloma P3X63Ag8U1 cells (4) (kindly provided by T. Watanabe, Medical Institute of Bioregulation, Kyushu University) by using polyethylene glycol 1000. The hybridomas were selected in HAT medium (RPMI 1640 medium contain-

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ing 10% fetal calf serum, 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine) and screened for reactivity with Kawasaki antigen by ELISA, and positive hybridomas were cloned by single-cell manipulation and cultivated on a feeder layer of mouse thymus cells. Then, 10^7 hybridoma cells were injected intraperitoneally into BALB/c mice which had received prior injection with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane), and the ascitic fluids were collected on days 10 to 14. The ascitic fluids were centrifuged at 6,600 × *g* for 20 min, and supernatants were partially purified by chromatography by using a Sephacryl S-300 column (Pharmacia Fine Chemicals, Uppsala, Sweden) and used as MAb preparations. Immunoglobulin classes and subclasses of the MAbs were determined by double immunodiffusion tests by using anti-mouse immunoglobulin G1 (IgG1), IgG2a, IgG2b, IgG3, IgM, and IgA antibodies prepared in rabbits (Miles Laboratories, Elkhart, Ind.).

IF. Indirect IF was performed as described previously (6, 14). The secondary antibodies used were fluorescein isothiocyanate-conjugated goat IgGs against (i) mouse IgM (μ-chain specific), (ii) the Fc fragment of mouse IgG (γ-chain specific), (iii) human IgM (μ-chain specific), and (iv) the Fc fragment of human IgG (γ-chain specific), all purchased from Tago, Inc. (Burlingame, Calif.).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The detailed procedures for SDS-PAGE and immunoblotting were essentially the same as those described previously (8). The resolving gel contained 10% polyacrylamide. The rickettsiae were purified by Percoll (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) density gradient centrifugation (10) and solubilized in sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 5 mM EDTA, 0.001% bromophenol blue, 0.065 M Tris hydrochloride buffer, pH 6.8) at 100°C for 10 min. After electrophoresis, the gel was stained with Coomassie brilliant blue. The secondary antibody used for immunoblotting was horseradish peroxidase-conjugated anti-mouse IgG (heavy- and light-chain specific) antibody prepared in rabbits (Cappel Laboratories, Cochranville, Pa.).

Inhibition ELISA. (i) Preparation of peroxidase-labeled MAb. A Kawasaki strain-specific MAb (no. 358) was labeled with horseradish peroxidase type VI (Sigma, St. Louis, Mo.) by the method of Yamada et al. (12). Prior to use, the peroxidase-labeled MAb was diluted to a concentration of 8 μg of protein per ml in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 0.1% bovine serum albumin (PBSTB).

(ii) Preparation of rickettsial antigen. Infected cells in a monolayer in a 500-ml culture bottle were scratched off by gently shaking with 8-g glass beads (3-mm diameter) into a small amount of culture medium. This suspension, which contained partially disrupted cells, was used as the antigen for the test without further purification. An appropriate concentration of the antigen for the test was determined in a preliminary test as described below.

(iii) Procedures of the inhibition ELISA. (a) Step 1. Each well of a microplate (Greiner, Nürtingen, Germany) was coated with 50 μl of a Kawasaki strain-specific MAb (no. 417; 12.5 μg of protein per ml) and was incubated overnight at 4°C. Then, the plate was washed three times with PBSTB, incubated with 10% bovine serum albumin in PBS for 2 h at 37°C, and washed again three times with PBSTB.

(b) Step 2. The antigen suspension (25 μl) was mixed with 25 μl of properly diluted test sera and 25 μl of PBSTB, and this mixture (75 μl) was added to the wells of the microplate. After incubation for 2 h at 37°C, each well was washed three times with PBSTB.

(c) Step 3. Fifty microliters of the peroxidase-labeled MAb was added to the wells, and the microplate was incubated for 2 h at 37°C, followed by three washings with PBSTB.

(d) Step 4. Freshly prepared substrate (100 μl), which consisted of 0.08% 2,2-azino-di-(3-ethylbenz-thiazoline-6-sulfonic acid) diammonium salt (5) and 0.006% hydrogen peroxidase in 0.05 M citrate-phosphate buffer (pH 5.0), was added to each well, and the microplate was incubated for 1 h at room temperature. The enzymatic reaction was stopped by adding 50 μl of 1.25% sodium fluoride, and the degree of color development was measured by reading the optical density (OD) at 415 nm by using a spectrophotometer.

(iv) Calculation of the percent inhibition. The percent inhibition was calculated by the following formula: inhibition (%) = [(negative control OD − sample OD)/(negative control OD − positive control OD)] × 100, in which negative control OD, positive control OD, and sample OD denote the optical density of the color developed when the antigen was mixed with antibody-negative sera, with a Kawasaki strain-specific MAb (no. 358), and with test sera, respectively. When the sera obtained from 10 patients from whom *R. tsutsugamushi* Kawasaki was isolated were examined by the inhibition ELISA, all the sera exhibited inhibition of greater than 70% (data not shown). On the other hand, when the sera obtained from 100 healthy persons who showed negative reactivities in IF were examined by inhibition ELISAs, none showed inhibitory properties, with inhibition being less than 30% (data not shown). From these results, percent inhibition above 70 was considered to be antibody positive and percent inhibition below 30 was considered antibody negative. Percent inhibition between 30 and 70 was judged as plus-minus.

Sera and antibodies. The sera used in the present study were obtained from the patients in Miyazaki prefecture in 1985 and 1986 and in Kanagawa prefecture in 1987. The MAbs against Gilliam, Karp, and Kato strains were the same as those used in our previous study (6).

RESULTS

Characterization of MAbs. Fourteen MAbs to the Kawasaki strain were obtained in the present study, and the immunoglobulin classes and the reactivities of six strains of *R. tsutsugamushi* were determined by IF (Table 1). These 14 MAbs were classified into five groups on the basis of analysis of cross-reactivities to the six strains as follows: Kawasaki strain specific (group 1); cross-reactive with both Kawasaki and Gilliam strains (group 2); cross-reactive with the Kawasaki and Kuroki strains, with one of the MAbs (no. 153) also reactive with the Shimokoshi strain (group 3); reactive with four strains (Gilliam, Karp, Kato, and Kawasaki) with low or no reaction with the Kuroki and Shimokoshi strains (group 4); and cross-reactive with all the strains (group 5).

In immunoblotting analyses using these MAbs (Fig. 1 and Table 1), the MAbs of groups 1 and 2 reacted with the 54- to 56-kDa polypeptides of Kawasaki or of Kawasaki and Gilliam strains, the MAbs of groups 3 and 5 reacted with the 46- to 47-kDa polypeptides of 2 or more strains, and the MAb of group 4 reacted with the 21- to 25-kDa polypeptides of the 4 strains. The patterns of cross-reactivity of the MAbs in IF and immunoblotting were relatively well correlated, except that the MAb of clone no. 3 in group 2 reacted strongly with both Kawasaki and Gilliam strains in IF but very weakly with the Gilliam strain in immunoblotting analysis and that

TABLE 1. Properties of MAbs obtained

MAb clone	Immunoglobulin class	IF titer against[a]: G	KP	KT	S	KW	KR	Polypeptide detected[b]
15	G3	<20	<20	<20	<20	2,560	<20	ND
87	G2b	<20	<20	<20	<20	20,480	<20	56K
188	M	<20	<20	<20	<20	320	<20	ND
193	M	<20	<20	<20	<20	2,560	<20	ND
296	G2a	<20	<20	<20	<20	40,960	<20	56K
358	G2a	<20	<20	<20	<20	40,960	<20	56K
417	G2b	<20	<20	<20	<20	20,480	<20	56K
3	G2b	20,480	<20	<20	<20	40,960	<20	56K
406	G2b	40,960	<20	<20	<20	40,960	<20	56K
134	G3	<20	<20	<20	<20	1,280	1,280	46K
153	M	<20	<20	<20	1,280	5,120	2,560	46K
11	G3	5,120	5,210	2,560	80	10,240	<20	25K
52	G1	320	320	320	640	640	640	46K
111	M	320	320	320	80	1,280	320	46K

[a] The letters indicate the rickettsial strains: G, Gilliam; KP, Karp; KT, Kato; S, Shimokoshi; KW, Kawasaki; KR, Kuroki. The titers are expressed as the reciprocal of the highest dilution of the MAb which gave positive reactions.
[b] Polypeptide detected by MAb immunoblottings. ND, Bands were not detected by immunoblotting analysis.

the MAb of clone 153 in group 3 did react with the Shimokoshi strain in IF but not in immunoblotting analysis.

Inhibition ELISA. Prior to performing the inhibition ELISA, we examined the conditions. When serially diluted crude Kawasaki strain antigens were adsorbed onto the MAb-coated wells and reacted with the peroxidase-labeled MAb (no. 358), the degree of color development decreased linearly with decreasing amounts of antigen (serial dilutions). For the inhibition ELISA, an antigen concentration that gave an OD of 1.0 to 1.5 after reaction with the peroxidase-labeled MAb was chosen. When rickettsial strains of other antigenic types were used instead of the Kawasaki strain, no color development was observed.

In the inhibition ELISA, MAbs 417 (group 1) and 3 (group 2) showed clear inhibition depending upon the antibody concentrations (Fig. 2). The other MAbs in groups 1 and 2 also showed similar inhibition profiles. This result indicates that the MAbs in groups 1 and 2 may recognize the same epitope or a closely associated epitope(s) that is recognized by the MAb (no. 417) used for coating the wells. On the other hand, the MAbs in groups 3 to 5 did not show inhibition (although only MAbs 153, 11, and 52 are shown in Fig. 2,

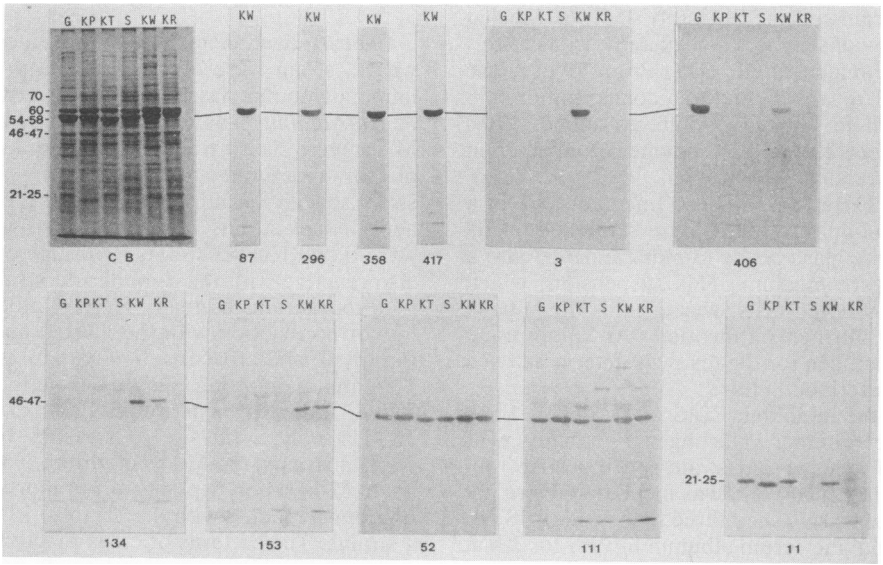

FIG. 1. Immunoblotting profiles of rickettsial polypeptides separated by PAGE with MAbs against the Kawasaki strain. The letters at the top of each lane indicate the rickettsial strain. The abbreviations are the same as those defined in Table 1, footnote *a*. Each panel was stained with Coomassie blue (CB) or allowed to react with MAbs (indicated with clone numbers), as shown at the bottom. The numbers at the left indicate the molecular sizes (in kilodaltons) of the bands determined in comparison with the migration of standard proteins.

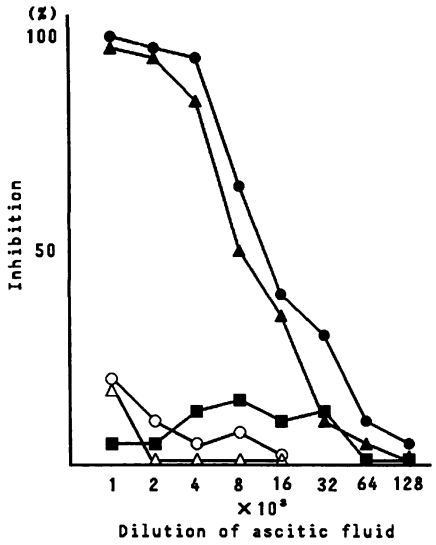

FIG. 2. Inhibition profiles of serially diluted MAbs 417 (●), 3 (▲), 153 (■), 52 (○), and 11 (△).

other MAbs in groups 3 to 5 did not show inhibitory properties either), indicating that the MAbs in groups 3 to 5 did not compete with the MAb (no. 417) used for coating.

Inhibition ELISAs were performed by using patients' sera, and the results were compared with those of IF titrations (Table 2). The patients' sera that were strongly reactive with the Kawasaki strain in IF showed clear positive results in the inhibition ELISA. Furthermore, sera from patients in the acute phase of tsutsugamushi disease, in which the IF titers were still low, appeared to be positive by the inhibition ELISA. The patients' sera that were weakly reactive with the Kawasaki strain but strongly reactive with other strains in IFs, such as the sera of the patients 2 and 4, showed negative or plus-minus reaction in the inhibition ELISAs. The patients' sera reactive with none of the strains in IF (from patients 5 and 8) were also negative in inhibition ELISAs. Taken together, these results indicate that (i) if the patients are infected with *R. tsutsugamushi* Kawasaki or with closely related strains, the inhibition ELISA will show clear positive results and (ii) this method is useful to estimate the serotype of rickettsiae which infected the patients, especially in the acute phase of tsutsugamushi disease.

TABLE 2. IF titers and percent inhibition of patients' sera obtained in Miyazaki and Kanagawa prefectures

Patient no.[a]	Days after onset of the disease[b]	Immunoglobulin class	IF titer against[c]:						ELISA inhibition (%)[d]
			G	KP	KT	S	KW	KR	
1	5	M	40	<20	<20	<20	160	<20	99
		G	<20	<20	<20	<20	80	<20	
	15	M	1,280	80	160	80	≥2,560	80	100
		G	1,280	20	20	20	≥2,560	20	
2	7	M	<20	<20	<20	<20	<20	<20	15
		G	20	20	320	20	<20	640	
	20	M	<20	<20	<20	<20	<20	<20	0
		G	320	320	640	320	40	≥2,560	
3	X	M	<20	<20	<20	<20	20	<20	97
		G	<20	<20	<20	<20	<20	<20	
	X + 14	M	640	40	160	40	≥2,560	40	100
		G	40	40	40	40	320	40	
4	7	M	80	320	160	40	40	1,280	32
		G	80	160	160	80	80	640	
5	8	M	<20	<20	<20	<20	<20	<20	0
		G	40	20	20	40	40	40	
	28	M	<20	<20	<20	<20	<20	<20	12
		G	40	20	20	40	40	40	
6	8	M	40	20	20	<20	640	<20	96
		G	<20	<20	<20	<20	<20	<20	
	25	M	640	20	80	20	1,280	40	99
		G	80	40	40	40	320	40	
7	3	M	160	<20	<20	20	640	<20	97
		G	<20	<20	<20	<20	80	<20	
	12	M	640	40	20	40	1,280	20	98
		G	40	40	40	<20	640	<20	
8	X	M	<20	<20	<20	<20	<20	<20	0
		G	<20	<20	<20	<20	<20	<20	

[a] Patients 1 through 4 were from Miyazaki prefecture, and patients 5 through 8 were from Kanagawa prefecture.
[b] Data represent number of days after the onset of disease that titers were determined. X, Day of onset of the disease was unknown.
[c] Abbreviations are the same as those in Table 1, footnote *a*. Titers are expressed as the reciprocal of the highest dilution of the sera which gave positive reactions.
[d] The patients' sera used were diluted to 1:10.

DISCUSSION

The presence of strain- or type-specific epitopes on the 54- to 56-kDa polypeptides located on the surface of *R. tsutsugamushi* was first reported by us (8), and this was confirmed by immunoblotting analyses using strain-specific MAbs (1, 3, 6). In our previous study, the Kawasaki strain was defined as a new antigenic type of *R. tsutsugamushi* distinguishable from the prototype strains Gilliam, Karp, and Kato by antigenic analyses using polyclonal and monoclonal antibodies (14). However, we recognized in the previous study some degree of cross-reactivity between the 54- to 56-kDa polypeptides of the Kawasaki and of the Gilliam strains in immunoblotting analysis using guinea pig hyperimmune sera. These observations are supported by the present study, in which two kinds of MAbs were obtained: one is specific to the 54- to 56-kDa polypeptide of the Kawasaki strain and the other is cross-reactive with the 54- to 56-kDa polypeptides of both Kawasaki and Gilliam strains, suggesting that the 54- to 56-kDa polypeptide of the Kawasaki strain contains both antigenic sites.

The 46- to 47-kDa and 21- to 25-kDa polypeptides are also located on the rickettsial surface (8). The 46- to 47-kDa polypeptide was first recognized as a common antigen among rickettsial strains in immunoblotting analyses using polyclonal antibodies (8). However, some polyclonal antisera and patients' sera occasionally showed different intensities of reactivity with the 46- to 47-kDa polypeptides of each strain (7, 8, 14). The fact that two kinds of MAbs against the 46- to 47-kDa polypeptide were obtained in the present study may correspond to these previous observations and suggests that the 46- to 47-kDa polypeptide may contain both common and subgroup-specific antigenic sites. The 21- to 25-kDa polypeptide could not be detected in immunoblotting analysis using guinea pig hyperimmune sera (8), but some patients' sera reacted with the 21- to 25-kDa polypeptide, showing subgroup specificity (7). In our unpublished studies, a MAb which was prepared to the Karp strain reacted with the 21- to 25-kDa polypeptides of the Gilliam, Karp, Kato, and Kawasaki strains but not with the 21- to 25-kDa polypeptide of the Shimokoshi strain (the Kuroki strain was not tested). The MAb of clone 11 in the present study showed similar reactivity, and we could thus confirm the subgroup-specific antigenicity of the 21- to 25-kDa polypeptide.

In inhibition ELISAs, all the MAbs in groups 1 and 2 which are reactive with the 54- to 56-kDa polypeptide showed clear inhibition, whereas the MAbs in the other groups which recognize the 46- to 47-kDa and 21- to 25-kDa polypeptides did not. The results indicate that this test is specific in that the antibodies which show positive reactivities recognize the same or sterically closely associated epitopes that are recognized by the MAb used for coating the wells.

The sera obtained from the patients who were suspected to have been infected with *R. tsutsugamushi* Kawasaki in IF analysis showed clear positive reactivities in ELISA inhibition tests, even at the early stages of the disease, when IF titers were still very low or negligible. The results suggest that the inhibition ELISA is more sensitive than IF analysis and that it is useful for serodiagnosis of the acute phase of the disease.

On the other hand, the other patients' sera that showed high IF titers to the Kuroki strain and very low or negligible IF titers to all the other strains appeared to be negative in inhibition ELISAs. The discrimination between positive and negative in inhibition ELISAs was very clear. As mentioned above, the antibodies which show positive reactivities recognize the same (or closely associated) epitopes that are recognized by the MAb used for coating the wells. Since we used a Kawasaki strain-specific MAb for coating the wells in the present study, infections of *R. tsutsugamushi* Kawasaki were detected by this test. Although the Kawasaki type strain was first isolated in Miyazaki prefecture and was found to be widely distributed in the Kyushu area (13), the present study clearly demonstrated the existence of infections of *R. tsutsugamushi* Kawasaki in other infected areas, such as Kanagawa prefecture.

Finally, crude antigen can be used in inhibition ELISAs. This may be another advantage of this test.

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