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Analysis of Antigenic Characteristics of *Rickettsia tsutsugamushi* Boryong Strain and Antigenic Heterogeneity of *Rickettsia tsutsugamushi* Using Monoclonal Antibodies

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Twenty-four monoclonal antibodies were produced by immunizing BALB/c mice with Rickettsia tsutsugamushi Boryong strain and used for the analysis of antigenic characteristics of R.tsutsugamushi Boryong strain and antigenic heterogeneity of R.tsutsugamushi by indirect immunofluorescent(IF) test. R. tsutsugamushi Kato, Karp, Gilliam, TA686, TA716, TA763, TC586, TH1817, and Boryong were used for the analysis of antigenic heterogeneity of R.tsutsugamushi. Five monoclonal antibodies were reactive with 27-kDa protein, four monoclonal antibodies were reactive with 47-kDa protein, and eight monoclonal antibodies were reactive with 56-kDa protein of R.tsutsugamushi Boryong strain. The reactive protein of seven monoclonal antibodies could not be identified by immunoblotting method. All monoclonal antibodies to 27-kDa protein and three monoclonal antibodies to 47-kDa protein, and five monoclonal antibodies to 56-kDa protein were reactive with three to eight strains among nine strains of R. tsutsugamushi tested. One monoclonal antibody reactive to 47-kDa protein(KI18) and two monoclonal antibodies reactive to 56-kDa protein(KI36, and KI37) reacted with all the strains of R. tsutsugamushi tested. Strain-specific monoclonal antibody(KI58) could be found among antibodies which were reactive with 56-kDa protein. There was no strain which showed same reactivity pattern to these 24 monoclonal antibodies among nine strains.

From this results, it could be concluded that Boryong strain is antigenically different from other strains of R.tsutsugamushi and antigenic heterogeneity of R.tsutsugamushi is due to the antigenic diversity of several proteins of R. tsutsugamushi including 56-kDa protein.

Key Words: *Rickettsia tsutsugamushi,* Boryong, antigenic heterogenicity

INTRODUCTION

Rickettsia tsutsugamushi, the causative agent of scrub typhus fever or tsutsugamushi disease, is antigenically diverse(Shirai et al.,1982) and several antigenic variants are known, such as representative strains Gilliam, Karp, Kato, and other antigenic types isolated in Thailand(Shirai et al.,1982;

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Takada et al.,1984; Tamura et al.,1985; Tamura et al.,1984). However, detailed characteristics of the antigenic heterogeneity and the relationship between antigenic diversity and the degree of virulence remained to be investigated(Murata et al., 1986).

The study of antigenic heterogeneity of *R.tsutsugamushi* at molecular level has been difficult, since polyclonal antibody raised in animals were used for serotyping and antigenic analysis. Attempts have been made to identify strains by using monoclonal antibodies which react with strain-specific epitopes which locate on the one class of envelope proteins(Murata et al.,1986; Oaks et al.,1989; Ohashi et al.,1992; Chang et al.,1990). These studies have shown that there are strain-specific epitopes and group-specific epitopes on 56-kDa polypeptides. Other polypeptide antigens with molecular weights of 150-, 110-, 70-, 47-kDa have been also described, but interstrain antigenic relatedness is not known.

Recently, we isolated a strain(Boryong) from a patient at Boryong Prefecture in Korea which has different antigenic characteristics from other prototype strains of *R.tsutsugamushi*(Chang et al., 1990). In this study, we produced 24 kinds of monoclonal antibodies by immunizing Boryong strain and analyzed the antigenic characteristics of Boryong strain and antigenic heterogeneity of *R.tsutsugamushi.*

MATERIALS AND METHODS

Culture and purification of Rickettsiae.

The *R.tsutsugamushi* used were Boryong(B119 previously, Chang et al., 1990), Karp(ATCC VR-150), Kato(Niigata), Gilliam(ATCC VR-312), TA686, TA716, TC586, TA678, TA763 and TH1817. L-cells were provided by NIH, Korea and cultivated in monolayers. Rickettsiae were grown in L-cells as described by Murata et al(1986). When a heavy growth of *R.tsutsugamushi* was observed in many cells by indirect immunofluorescent microscopy, cells were harvested. The cells were washed three times and resuspended in TS buffer(33mM Tris, 250mM sucrose, pH 7.4) and homogenized by Potter-Elvejhem homogenizer. The homogenates were centrifuged at 300 X g for 5 min and the supernatants were used for purification of *R.tsutsugamushi. R.tsutsugamushi* were purified by using percoll density gradient centrifugation method as described by Tamura(Tamura et al.,1982).

Indirect immunofluorescent(IF) test.

If test was done as described by Tamura et al. with some modifications(1984). L-cells infected with each strain of *R.tsutsugamushi* were resuspended with EMEM and 10ul of each suspension was applied to microscopic spot slides. The spots were dried at room temperature for at least 1 hr and then fixed with acetone for 10 min. The slides were stored at -70°C until use. Culture supernatant of each hybridoma or ascitic fluid were serially diluted with phosphate buffered saline(10mM phosphate, pH 7.4; PBS) and 20ul of each monoclonal antibody solution was applied to the spots. The slides were incubated at 37°C for 30 min in moist chambers and washed three times with PBS. 20ul of fluresceín conjugated anti-mouse IgG were applied to the spots and incubated at 37°C in a moist chamber for 30 min. The slides were washed three times with PBS and observed.

Monoclonal antibody production.

Six to eight week old BALB/c mice were injected with *R.tsutsugamushi* Boryong strain subcutaneously. When mice became seriously ill, tetracycline was injected subcutaneously. Three weeks later, the mice were injected with *R.tsutsugamushi* Boryong strain again. Three days after the second injection, mice were sacrificed and spleen cells from the mice were fused with P3x63Ag8.V653 myeloma cells(1:1, cell to cell ratio) by using polyethylene glycol 1500(Boerhinger Mannheim) as the fusion agent as described by Chang et al (1990). After incubation for 18hr at 37°C in the atmosphere of 5% CO_2 and 100% relative humidity, 0.1 ml of selective medium was added to each well. Thereafter selective medium were changed at 72hr interval. The supernatants of wells with viable growth were screened for immunoglobulin by IF test. Ascites were produced by injecting BALB/c mice with $5X10^6$ cloned cells intraperitoneally. Ammonium sulfate were added to the ascitic fluids(final 50%)to precipitate globulin fraction.

SDS-PAGE and immunoblotting.

SDS-PAGE was done by a modification of the Laemmli method (Laemmli, 1970). The separation gel contained 8% polyacrylamide, 0.26% bisacrylamide and 0.1% SDS in 0.37M Tris buffer and polymerized by the addition of tetramethylenediamine and ammonium, persulfate at final concentrations of 0.3% and 0.066%. Purified rickettsiae

Table 1. The reactivity of 17 monoclonal antibodies to 9 strains of *R.tsutsugamushi* and molecular weight of their reactive proteins

Monoclonal antibody	Molecular weight* of reactive protein	Strains								
		Boryong	Karp	Kato	Gilliam	TA686	TA716	TA763	TC586	TH1817
KI17	27-kDa	+	-	-	+	+	+	+	+	+
KI34	27-kDa	+	-	+	-	+	+	-	+	+
KI41	27-kDa	+	-	-	-	-	-	+	-	+
KI43	27-kDa	+	-	-	-	-	+	+	-	+
KI54	27-kDa	+	-	+	-	+	+	-	+	+
KI18	47-kDa	+	+	+	+	+	+	+	+	+
KI20	47-kDa	+	-	-	+	+	+	+	-	-
KI24	47-kDa	+	+	+	+	+	+	+	-	+
KI29	47-kDa	+	-	-	+	+	+	+	-	-
KI11	56-kDa	+	-	+	-	+	-	+	-	-
KI13	56-kDa	+	-	-	-	-	+	+	-	-
KI16	56-kDa	+	+	-	-	-	+	+	+	+
KI30	56-kDa	+	-	+	-	-	+	+	+	
KI36	56-kDa	+	+	+	+	+	+	+	+	+
KI37	56-kDa	+	+	+	+	+	+	-	+	+
KI39	56-kDa	+	-	+	-	-	-	+	+	-
KI58	56-kDa	+	-	-	-	-	-	-	-	-

* Molecular weight of reactive proteins were determined by immunoblotting using R.tsutsugamushi Boryong strain as an antigen(Fig. 1).

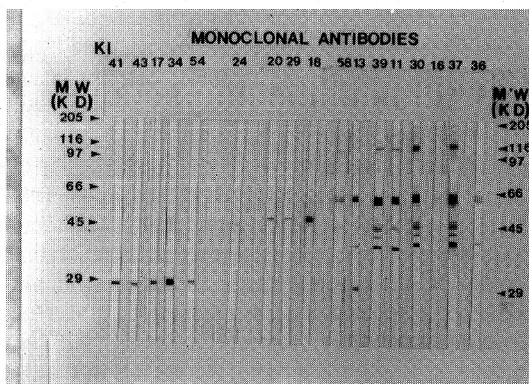

Fig. 1 Immunoblotting analysis of monoclonal antibodies produced by immunizing *R.tsutsugamushi* Boryong strain.

were solubilized in 0.1M Tris buffer(pH6.8) containing 2% SDS, 5% mercaptoethanol, 20% glycerol and 0.001% bromphenol blue by heating at 100oC for 10 min, and subsequently applied to sample wells. The samples were electrophosed at constant voltage(70V) in 0.025 Tris buffer(pH8.3) containing 0.1% SDS and 0.192M glycine for 2 hrs. Polypeptide bands were visualized by fixing the gel with a mixture of methanolacetic acid and distilled water(4:1:4,v/v) for 30 min at room temperature and staining the gel with 0.25% Coomassie blue R250 solution for 1 hr. Immunoblotting was performed by modifications of the method of Towbin et al(Towbin et al.,1992).

RESULTS

Characterization of monoclonal antibodies.

Supernatant fluids of 10 to 15 day cultures of hybridomas were screened for *R.tsutsugamushi* specific antibody by IF test and 24 monoclonal antibody were found. The molecular weights of their reactive polypeptides were analyzed by immunoblotting with Boryong strain. Of total 24 monoclonal antibodies produced, five monoclonal antibodies were reactive with 27-kDa protein(20.8%), one monoclonal antibody was reactive with 43-kDa protein(4.1%), three monoclonal antibodies

Table 2. The reactivity of 7 monoclonal antibodies* to 9 strains of *R.tsutsugamushi*

Monoclonal antibody	Strains								
	Boryong	Karp	Kato	Gilliam	TA686	TA716	TA763	TC586	TH1817
KI12	+	-	-	-	-	-	-	-	-
KI14	+	+	-	-	-	-	-	-	+
KI15	+	-	-	-	-	-	-	-	-
KI20	+	-	-	+	+	+	+	-	-
KI23	+	-	+	-	+	-	+	-	-
KI24	+	+	+	+	+	+	-	-	-
KI31	+	+	+	+	+	+	+	+	+

* The molecular weight of the reactive proteins could not be identified by immunoblotting method.

were reactive with 47-kDa protein (12.5%), and eight monoclonal antibodies were reactive with 56-kDa protein(33.3%). The reactive proteins of 7 monoclonal antibodies(29.1%) could not be detected by immunoblotting method. The characteristics of monoclonal antibodies are shown in Fig.1.

Comparison of antigenicity of Boryong strain with other eight strains of *R.tsutsugamushi*.

For the characterization of antigenicity of Boryong strain, the reactivity of *R.tsutsugamushi* Boryong strain and other 8 strains to 24 monoclonal antibodies were analyzed by IF test. The results are summarized in Table 1 and Table 2. Two strain-specific monoclonal antibodies(KI58, KI12) to *R.tsutsugamushi* Boryong strain were found. KI58 monoclonal antibodies which reacted with 56-kDa protein reacted with *R.tsutsugamushi* Boryong strain specifically, indicating that strain-specific epitopes of *R.tsutsugamushi* located on this molecule (Table 1). However the reactive protein of the other strain-specific monoclonal antibody(KI12) could not be identified by immunoblotting(data not shown). It is presumed that the epitope which reacts with KI12 monoclonal antibody might be denatured during the sample preparation for the SDS-PAGE. KI18 monoclonal antibody reactive with 47-kDa protein, KI36 monoclonal antibodies reactive with 56-kDa protein and KI31 monoclonal antibody of which reactive protein could not be identified by immunoblotting reacted with all the strains of *R.tsutsugamushi* tested in this experiment. The other 19 monoclonal antibody were reactive to several strains of *R.tsutsugamushi*, but the reactivity patterns to nine strains were different from each other. KI41 monoclonal antibody and KI15 monocloanl antibody reacted with only two strains of *R.tsutsugamushi* among 9 strains tested, while KI24 monoclonal antibody reacted with all the strain tested except TC586 strain(Table 1).

There was no strain which showed same reactivity patterns to these 24 monoclonal antibodies with that of Boryong strain. This result indicated that Boryong strain is antigenically different from other prototype strains. These results also showed that the antigenic differences of *R.tsutsugamushi* could be found not only on 56-kDa protein, but also on 27-kDa protein and 47-kDa protein.

DISCUSSIONS

It was well known that *R.tsutsugamushi* is antigenically heterogeneous(Takada et al.,1984; Shirai et al.,1982; Tamura et al.,1985; Tamura et al., 1984). And it has been suggested that the virulence of *R.tsutsugamushi* and the vector specificity of *R.tsutsugamushi* might be related with the antigenicity of *R.tsutsugamushi*(Oaks et al.,1989; Oaks et al.,1987; Ohashi et al.,1992). In recent years, a number of strains which are antigenically different from Karp, Kato, and Gilliam strains were isolated in Thailand and Japan(Tamura et al.,1985; Tamura et al.,1984; Yamamoto et al.,1986; Yamashita et al.,1988). It has been reported that the strain-specific epitope of *R.tsutsugamushi* located on 56-kDa protein and 120-kDa protein, but the detailed characteristics of antigenic heterogeneity of *R.tsutsugamushi* still remained to be determined(Murata et al.,1986).

Recently, we isolated a antigenically new strain of *R.tsutsugamushi* from a patients at Boryong Prefecture in Korea(Chang et al.,1990). In this study, we produced 24 monoclonal antibodies by immunizing *R.tsutsugamushi* Boryong strain and

used for the characterization of the antigenic structure of Boryong strain and the analysis of antigenic heterogeneity of *R.tsutsugamushi*.

The reactivity pattern of Boryong strain to 24 monoclonal antibodies was different from those of other eight strains of *R.tsutsugamushi* and we could find two monoclonal antibodies which were specific to *R.tsutsugamushi* Boryong strain(KI58 and KI12). KI58 monoclonal antibody, a Boryong specific monoclonal antibody, reacted with 56-kDa protein of *R.tsutsugamushi* Boryong strain. These results confirmed that *R.tsutsugamushi* Boryong strain is antigenically different from other strains of *R.tsutsugamushi* and 56-kDa protein of *R.tsutsugamushi* has strain-specific epitopes(Murata et al., 1986).

Little is known about the antigenic diversity of 27-kDa protein of *R.tsutsugamushi*. In this study, the reactivity patterns of nine strains of *R.tsutsugamushi* to 27-kDa protein reactive monoclonal antibodies(KI17, KI34, KI41, KI43, and KI54) revealed that there are significant differences in the antigenicity of 27-kDa proteins among strains. Karp strain did not react with any monoclonal antibodies which are reactive with 27-kDa protein, while Gilliam strain reacted with one monoclonal antibody(KI17) and Kato strain with two monoclonal antibodies(KI34 and KI54). *R.tsutsugamushi* strains isolated in Thailand reacted with three to four 27-kDa protein reactive monoclonal antibodies.

47-kDa protein of *R.tsutsugamushi* has been known as species specific antigen and there was little antigenic variations between strains(Oaks et al.,1989; Oaks et al.,1987). In this study, we could find one 47-kDa protein reactive monoclonal antibody(KI18) which were reactive with all the strains of *R.tsutsugamushi*, supporting that 47-kDa protein has species-specific epitopes. But we could find three monoclonal antibodies(KI20, KI24, and KI29) which did not react with some strains of *R.tsutsugamushi*. All antibodies to 47-kDa protein reacted with Gilliam, TA686, TA716, and TA763 strain. This results suggested that the antigenicity of 47-kDa protein also differs among strains.

KI58 monoclonal antibody reacted with *R.tsutsugamushi* Boryong strain only and we could confirm that 56-kDa protein of *R.tsutsugamushi* has strain-specific epitopes. But KI36 monoclonal antibody reacted with all the strains of *R.tsutsugamushi*, and other 56-kDa protein reactive monoclonal antibodies(KI11, KI13, KI16, KI30, KI37, and KI39) did not react with some strains.

From these results, it can be concluded that *R. tsutsugamushi* Boryong strain is antigenically different from other strains of *R.tsutsugamushi* and antigenic heterogeneity of *R.tsutsugamushi* is not entirely dependent on 56-kDa protein, although strain-specific epitopes could be found on 56-kDa protein.

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