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Antigenic Modification of Leptospira and Change in Susceptibility to Host Bactericidal Effects

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ABSTRACT **Variant strains, induced by co-cultivation of *Leptospira interrogans* serovar *copenhageni* strain Shibaura with its homologous antiserum in a liquid medium, showed reduced agglutinability to the antiserum in comparison with that of the original parent Shibaura strain. The reduction was inversely correlated to the concentration of the antiserum in the medium. Furthermore, these variants showed reduced agglutinability to anti-leptospiral lipopolysaccharide (LPS) serum and antileptospiral LPS monoclonal antibodies. The variants became resistant to complement-mediated and macrophage-mediated bactericidal effects in the presence of antibody to the LPS. The level of resistance was related to the degree of the reduced agglutinability. The clearance time of these variants from the blood of the mice was observed to be longer than that of the parent Shibaura strain when these bacteria were inoculated into the mice immunized with heat-inactivated parent Shibaura strain. It was concluded, therefore, that these variants acquired the ability to survive for a longer period *in vivo* than that of the parent organism. These results suggest that variation of Leptospires, which has been considered as a temporary modification of LPS, is one of the ways it escapes from the host defense mechanisms.**

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Key Words: Antigenic modification, Host defense, Leptospiral lipopolysaccharide, Monoclonal antibody, Variants

1. INTRODUCTION

Antigenic variation has been reported in leptospires[1-5]. These studies reported that the agglutinability of the variants was reduced when cultivated in a liquid medium containing homologous antiserum. The antigenic variation of leptospires was considered to be due to a temporary quantitative change of the surface antigen. An application of Laurell rocket immumoelectrophoresis to SDS-extracted antigens revealed the distinct quantitative difference of antigen between the parent and the variants[3]. The magnitude of the quantitative difference could be modified by concentrations of the homologous antiserum supplemented in the medium. The antigen mainly reduced was considered to be LPS component of the bacteria. In this study, we examined resistance of the variants to the antibody-mediated host defense mechanisms.

2. MATERIALS AND METHODS

2. 1 Strains

L. interrogans serovar *copenhageni* strain Shibaura was used for an original parent strain. The organism was grown at 30°C in 0.2% tryptose phosphate broth (Difco) supplemented with 10% inactivated rabbit normal serum and a small amount of phenol red as a pH indicator (growth medium). Solid serum medium with 1% Noble agar (Difco) was used to count the number of viable leptospires.

2. 2 Immune sera and monoclonal antibodies

Immune sera were obtained from two rabbits by the methods described previously[9]. Each rabbit was immunized with whole bacterial cells of strain Shibaura or leptospiral LPS[9]. Monoclonal antibodies, SCA (IgG_{2a}) and SCB (IgG_{2b}), which have been established to react with leptospiral lipopolysaccharide[8], were used for the present experiments.

2. 3 Immune serum medium

The immune serum medium was prepared according to the methods described previously[3]. In brief, various cocentrations of the rabbit anti whole cell serum were added to the growth medium. The concentrations of each immune serum were 0.006, 0.012, 0.025, 0.05, and 0.1%. The leptospires grown in each concentration of the immune serum were designated as 006V, 012V, 025V, 050V, and 100V, respectively.

2. 4 Isolation of clones from the cultivated leptospires grown in the immune serum medium

The solid immune serum medium was used to isolate the colonies, as described previously[3], from the culture of leptospires grown in a 0.1% immune serum medium. Leptospires inoculated into the solid immune serum medium were incubated at 30°C for 12 days. The colonies grown on the medium were randomly picked up and cultured in a liquid immune serum medium containing 0.1% anti-parent serum. Each colony was considered as one clone.

2. 5 Microscopic agglutination test

The test was performed as previously described[3].

2. 6 Leptospiricidal activity test

Antibody (anti-parent serum, anti-LPS serum, monoclonal antibody SCA) and complement (normal guinea pig serum) were used for the leptospiricidal activity test. The test was performed in the same manner as previously described[6].

2. 7 Phagocytosis assay *in vitro*

The phagocytosis was assessed by the modified method of Young *et al.*[7]. Details of it were described previously[8]. The standard mixture was consisted of 0.5 m*l* of the peritoneal macrophage suspended in Hank's balanced salt solution (pH 7.2, HBSS) containing 5×10^6 cells and 0.1 m*l* of leptospiral suspension containing 5×10^5 organisms in HBSS, 0.1 m*l* of complement (fresh normal mouse serum), 0.1 m*l* of rabbit antiparent serum diluted into subagglutinative concentration (1 : 200) by HBSS and 0.2 m*l* of HBSS.

2. 8 Immunization and clearance test

A total of 140 ddY mice (male, six-week-old) was used. The mice were divided into 4 groups. First 2 groups were immunized with 2×10^8 cells of heat-inactivated (60°C for 30 min) parent organisms. The other groups were inoculated with the same volume of the medium without the bacteria. After 1 week, 2 groups of the mice including each one of the immunized and nonimmunized group were injected with 2×10^8 viable cells of the strain Shibaura suspended in the growth medium. The remaining 2 groups of mice were injected with the same number of viable cells of the 100V strain through the tail veins. These mice were bled for sampling of the blood at 1, 15, 39, 63, 91, 139 and 187 hours after the

2nd injection of the organisms. The samples were immediately inoculated on the solid medium at suitable dilutions (phosphate buffer, pH 7.2) to count the number of viable organisms. The number of the organisms per m*l* was calculated from these data.

2. 9 Bacteriological examination of the kidneys

Bacteriological examination of kidneys was performed by the method previously described[6]. Briefly, the cortex of the kidney was collected from the infected mice 8 days after inoculation of the organisms. Three small pieces of the cortex were put into small tubes containing 2 m*l* of liquid normal serum medium.

2. 10 Statistical analysis

The data obtained from the present study were analyzed for statistical differences by unpaired t test.

3 RESULTS

3. 1 Reduction of the agglutinability in antigenic variants

The reduction of agglutinability was observed in the variants produced by co-culture of the Shibaura parent strain with the rabbit anti-parent antiserum (Table 1), and its level was dose-dependent in relation to the concentrations of the antiparent serum. The reduction of the agglutinability in these variants was similarly found against anti-LPS antiserum and monoclonal antibodies (Table 1). The reduced agglutinability was recovered to the levels of the parent after the variants were passed several times in the normal serum medium.

Fourteen colonies (clone 1-14, the origin was 100V strain) of the variant grown on the solid medium containing 0.1% anti-parent serum were examined by immunodiffusion using anti-parent and anti-variant sera. These clones were antigenically identical to each other and to the original variant, but different from the parent (data not shown). Levels of agglutinability of these cloned variants were similar to the original 100V strain (Table 1).

3. 2 Sensitivity of the variants to leptospiricidal test

Table 1 *Comparison of agglutinability among leptospires grown in the medium containing anti-parent serum and original parent Shibaura strain*

Leptospires	Concentration of anti-parent serum for cultivation(%)	Agglutination agaismt[a]				
		anti-parent serum	anti-LPS serum	mnoclonal antibodies[b]		
				SCA-A	SCA-S	SCB-S
100V 1-14[c]	0.100	128-256	16-32	16-32	—	—
100V	0.100	256	32	32	—	—
050V	0.050	512	64	64	8	—
025V	0.025	1024	64	128	16	—
012V	0.012	1024	256	512	16	2
006V	0.006	2048	512	1024	32	4
Parent	0	2048	1024	2048	32	8

a: Data are expressed as minimum dilution of anti-serum and monoclonal antibody in which bacterial growth was observed.

b: SCA—A: Ascites including IgG_{2a} antibody which recognizes leptospiral LPS of Icterohaemorrhagiae serogroup.

SCA—S: Surernatant from culture of SCA hybridoma.

SCB—S: Supernatant from culture of SCB hybridoma. SCB—S is including IgG_{2b} which recognizes leptospiral LPS of Icterohaemorrhagiae serogroup.

c: Fourteen clones isolated from 100V strain.

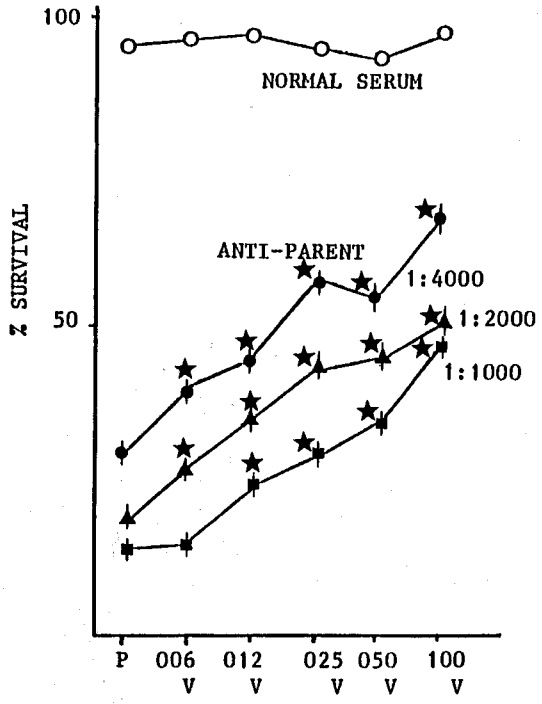

Fig. 1 Effect of anti-parent serum on serum-susceptibility of unstable variants. Standard deviations are indicted as bars. Asterisks show the significant difference against the value of parent.

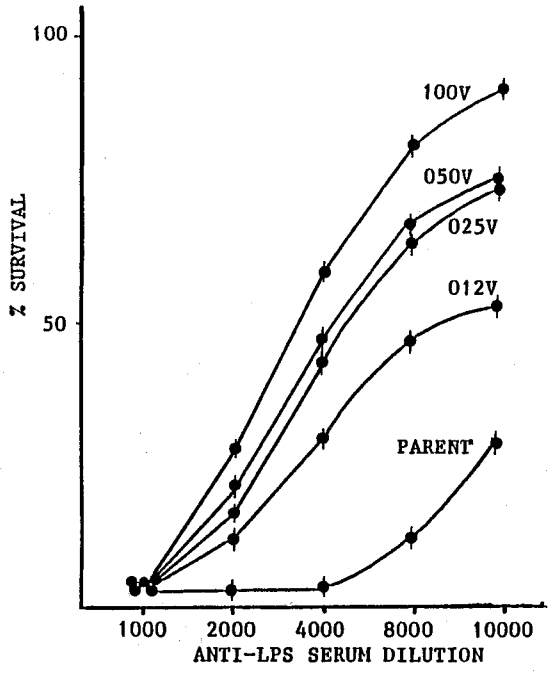

Fig. 2 Effect of anti-LPS serum on serum-susceptibility of unstable variants. Standard deviations are indicted as bars. All values obtained from 012V, 025V, 050V and 100V were significantly different from these of the parent.

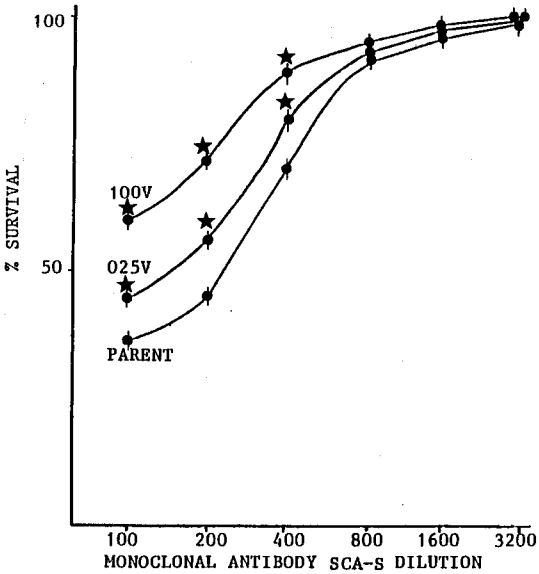

Fig. 3 Effect of a monoclonal antiboiy SCA-S on susceptibility of unstable variants. Standard deviations are indicted as bars. Asterisks show significant difference against the value of parent.

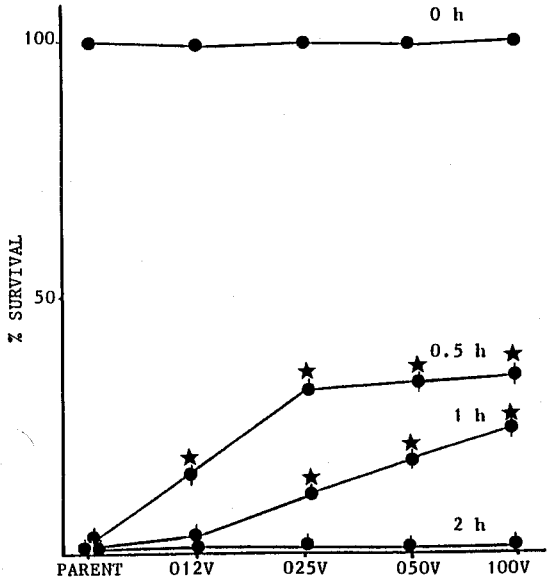

Fig. 4 Susceptibility of unstable variants to antibody mediated phagocytosis. Subagglutinative dilution of anti-parent serum was used for experiments. Standard deviations are indicted as bars. Asterisks show the significant difference against the value of parent.

As shown in Figure 1, the variants were more resistant than the parent in leptospiricidal test using anti parent serum and complement. Thus, the resistast level of the variants to the leptospiricidal effect was increased with the concentrations of anti-parent serum in the growth medium. Similarly, the variants were more resistant than the parent in the test using anti-LPS antiserum (Figure 2) and monoclonal antibody SCA-S (Figure 3).

3. 3 Antibody-mediated phagocytosis

As shown in Figure 4, both the parent and variants were inactivated by mouse peritoneal macrophages. The parent exhibited a more sensitive response. At 1 h incubation, the response was related to the concentration of anti-parent serum which was used for production of the variant strains.

3. 4 Clearance test

Figure 5 shows the results of clearance test of the parent and variant 100V from blood of mice. In immunized mice, the number of the parent organisms were rapidly decreased. None of the bacteria could be recovered from the blood after 15 hrs post infection. In contrast, the variant 100V was detected in the blood even after 15 hrs post infection. Additionally, the number of 100V was more than 100 times that of the parent after 1 h post infection. In non-immunized mice, both 100V and parent were cleared from the blood. However, the number of 100V in the blood was smaller than that of the parent.

The rate of renal infection of the parent and variant 100V was examined on the 8 th post infection day (Table 2). The percentages of renal infection were 80% (100V) and 100% (parent) in non-immunized mice and 27% (100V) and 0% (parent) in immunized mice.

4 DISCUSSION

The data presented here showed that the variants acquird resistance to leptospiricidal effects of anti-parent antibody plus complement and the antibody-mediated bactericidal effects by phagocytosis. The resistance was related to the activity that the variants could survive for longer period than the parent in the blood of mice immunized with the parent organism. The results clearly indicated that the variant of leptospires was one of the forms to escape from the host defense mechanisms.

Table 2 *Rate of renal infection*

Strains used	Rate (%) of renal infection in non-immunized mice		immunized mice	
Variant 100V	80(12/15)	NS	27(4/15)	**
Parent	100(15/15)		0(0/15)	

**: significant difference was observed ($P<0.05$).
NS: not significant

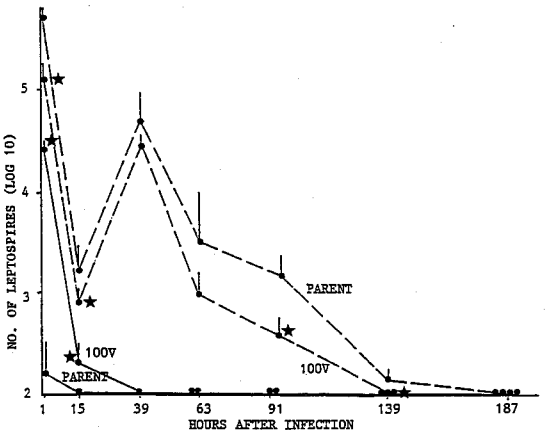

Fig. 5 Clearance test in non-immunized mice (----) and immunized mice (———). Standard deviations are indicted as bars. Asterisks show the significant difference against the value of parent at each experimental period.

Coverage of cell surface with LPS was considered to induce resistance against the host defense mechanisms. It has been reported that cultivation of leptospires in the presence of dipheylamine (DPA) induces a decrease in the content of leptospiral LPS[6]. The DPA-treated leptospires become susceptible to leptospiricidal effect of normal rabbit serum and phagocytes in association with this decrease of LPS. The unstable antigenic variants in this study also showed decrease of agglutinability against anti-LPS antiserum. However, they were not susceptible to leptospiricidal effects of normal serum or phagocytes. The resistance against bactericidal effect without antibody was different between the unstable variants and DPA-treated bacteria.

The unstable antigenic variation has been found in leptospires *in vitro*[1,4] and *in vivo*[2,5]. The variants which were isolated from *patoc* in the presence of heterologous immune serum against *semaranga* were reported to show reduced agglutinability. However, the reduction was not stable. Once, they were cultivated in normal serum medium for several passages, they resumed their initial antigenic characteristics[1]. Six serovars of *Leptospira interrogans*, which were adapted to grow in each homologous immune serum and were weakly agglutinated with the homologous immune serum, displayed their original antigenic characteristics when they were inoculated into a medium without the immune serum[4]. Similar unstable variants were easily discovered *in vivo*[2,5]. These previous studies have suggested that the unstable variation is a temporary antigenic modification. Our data supported these studies and suggested that the ability of leptospires to grow in extreme environments was accompanied by gross changes in membrane composition.

Organisms must fulfill several minimum requirements to cause disease. First, the bacterial agents must adhere on or invade into host tissues. They must resist various host defense mechanisms and grow in the tissues. The host, finally, are damaged by the effects of the agent and the disease occurs. Changes of the structural components, i. e. quantitative and/or qualitative changes of LPS, of the leptospiral envelope are important for pathogenicity of the organism.

5 CONCLUSIONS

The antigenicity of *L. interrogans* was modified when the bacteria were grown in a liquid medium containing a homologous antiserum. The modified portion on the cell surface was considered as lipopolysaccharide. The antigenic variants became resistant to antibody-mediated bactericidal effects and survived for longer period *in vivo* than the parent.

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レプトスピラの抗原変異と宿主の殺菌作用に対する感受性の変化

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Leptospira interrogans 血清型 *copenhageni* 芝浦株をホモの抗血清を含む液体培地で培養したところ，その凝集原性は培地に加えられた抗親株血清の濃度に依存して低下した．同様に抗リポ多糖血清あるいはリポ多糖に対する単クロン性抗体に対する凝集原性もこれらvariantでは低下した．リポ多糖に対する抗体の存在下における補体依存性の殺菌作用やマクロファージ依存性の殺菌作用に対し，variantは抵抗性となった．抵抗性の程度は凝集原性に関連していた．加熱不活化した親株を免疫後variantで攻撃したところ，これらは血中からゆっくり排除された．一方，親株は速やかに血中から排除された．このように，variantは生体内において親株より長い期間生存した．以上の結果は抗体存在下でのリポ多糖の一時的な変化は宿主の防御機構から本菌が逃避する一つの方法であることを示唆した．