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MUTATION OF GROWTH OF LEPTOSPIRES IN SHENBERG'S MEDIUM

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A procedure was first described in which a strain of *Leptospira icterohaemorrhagiae* (Shibaura), which could not be grown in SHENBERG's chemically defined medium even when the inoculum size was increased up to 2×10^9 leptospires per ml, began to grow in the same medium. This was attained only by using the mutant which can grow in the boiled serum medium. No selection was done in SHENBERG's medium so far as the mutant selected in the boiled serum medium was used. The mutant thus obtained was transferred in SHENBERG's medium, and the properties of the mutant were examined during the 22nd~25th transfers. Growth in the serum medium, antigenicity examined by the reciprocal microscopic agglutination test and immunodiffusion, and virulence for guinea pigs were the same for the parent and the mutant. However, the colonial size of the mutant was obviously smaller than that of the parent.

INTRODUCTION

Chemically defined media for cultivation of leptospires have been studied only recently. VOGEL & HUTNER reported a chemically defined medium in which several leptospiral strains grew. Serial subcultivation could be done only from large inocula (10 to 50%, v/v), and maximal densities were only one-tenth of those in serum media. The medium was modified by STALHEIM & WILSON but the growth of leptospirae was still not sufficient. JOHNSON & GARY described that fatty acids are important for metabolism of leptospires. On the basis of this fact, STALHEIM described another modified defined medium. SHENBERG described a defined medium whose composition was more simple than that of others but supported a good growth of leptospires. SHENBERG's medium permitted a good growth of 52 strains (9 serogroups) of the 61 strains (12 serogroups) tested, but did not permit growth of the remaining strains.

We wished to grow in SHENBERG's medium the strains which had been unable to grow in the same medium. For this purpose, attempts were made by the authors to obtain the mutant which can grow in SHENBERG's medium. This was attained only by using the mutant which can grow in the boiled serum medium[13]. The differences between the mutant and parent were also studied. These results are described below.

MATERIALS AND METHODS

Strains Virulent *L. icterohaemorrhagiae* Shibaura mainly used in the present experiment was provided by Dr. KITAOKA, WHO/FAO Leptospirosis Reference Laboratory, National Institute of Health, Tokyo. *L. icterohaemorrhagiae* TR-1 and Asakawa, *L. autumnalis* Akiyami A, Fujiwara and Tomoda, *L. hebdomadis*, Kyoto and Mikata, *L. australis* Akiyami C and Kimura were given by Dr. E. KITA, Chugoku branch laboratory, National Institute of Animal Health, Wadayama, Hyogo. *L. canicola* Hond Utrecht IV was received from Dr. T. FUJIKURA, National Institute of Animal Health, Tokyo.

Media Media used for cultivation of leptospires were as follows; 0.2% tryptose phosphate broth (Difco) containing 10% normal rabbit serum[1)] which was designated in this report as serum medium, the boild serum medium prepared according to YANAGAWA & WILSON, and a chemically defined medium recently reported by SHENBERG (SHENBERG's medium). The solid medium for colonial growth was prepared according to COX & LARSON; FUJIKURA's method of colony formation was followed. Phenol red was added to every medium as the pH indicator at a concentration of 0.0006%. Incubation was carried out at 30°C.

Counting of leptospiral cells Leptospiral cells were counted by darkfield microscopy by using the counting chamber described by YANAGAWA et al.[12)]

Estimation of leptospiral virulence The number of leptospires (*L. icterohaemorrhagiae* Shibaura, 7-day-old culture) was counted and the serial tenfold dilutions were then made so as to obtain the necessary number of leptospires per ml. One ml of the each diluent was inoculated into guinea pigs (about 250 g in weight) intraperitoneally. The death of the guinea pigs, along with the period of time which it took for them to die was the indicator of virulence.

Preparation of antiserum Rabbits (about 2.5 kg in weight) were immunized with the parent and mutant of *L. icterohaemorrhagiae* Shibaura respectively. The leptospiral culture (2 ml) heated at 56°C for 30 minutes was first injected. Then the serial injection was carried out increasing amounts of leptospires. Seven-day-old cultures of increasing amounts up to 40 ml cultures of the parent strain (in serum medium) and those up to 200 ml of the mutant strain (in SHENBERG's medium) were washed by centrifugation, suspended in 1~2 ml of 0.01 M phosphate buffer solution (pH 7.2), and injected into rabbits at 7-day-intervals for 5 weeks. On the fifth day after the last injection, each rabbit was bled and the sera obtained were stored at −20°C. Details of the parent and mutant were described in the text.

Concentration of antisera for immunodiffusion The rabbit antisera prepared as above were concentrated before using for immunodiffusion, because a preliminary immunodiffusion showed that the immune sera were not completely sufficient for the test. A saturated ammonium sulfate solution was added to the sera by drops, while the sera were shaken, to give a concentration of 40% of ammonium suflate. After centrifugation at 10,000 rpm for 20 minutes, the precipitates were suspended in phosphate-buffered saline (pH 7.2) and dialized against the same buffered saline. After dialysis, the final volumes of concentrated antisera were found to be one-fifth of the originals. All these procedures were done at 4°C.

Immunodiffusion The immunodiffusion method of OUCHTERLONY was used as

described by MURTY & HANSON with slight modification. The gel was prepared with 0.02 M phosphate buffer solution (pH 7.2). Antigens were extracted with 1% sodium deoxycholate (SDC)[11]. Leptospiral cultures were centrifuged at 12,000 rpm for 30 minutes, and the sedimented cells were suspended in 0.02 M phosphate buffer solution (pH 7.2) so as to contain 10^{10} cells per ml. The leptospiral cell suspensions were mixed with an equal amount of 2% SDC (final 1%). The mixtures were incubated and shaken at 45°C for 4 hours and centrifuged at 12,000 rpm for 30 minutes. The supernatants were used as antigens.

RESULTS

The growth of 11 strains in SHENBERG's medium was examined. The culture of each strain grown in the serum medium was inoculated into SHENBERG's medium so as to give 10^7 cells per ml. The results are shown in table 1. Eight strains grew up to 10^8 to 2×10^8

TABLE 1 *Growth of leptospires in SHENBERG's medium*

SEROTYPE	STRAIN	GROWTH
L. autumnalis	Akiyami A	+
	Fujiwara	+
	Tomoda	+
L. australis	Akiyami C	+
	Kimura	+
L. hebdomadis	Kyoto	+
	Mikata	+
L. canicola	Hond Utrecht IV	+
L. icterohaemorrhagiae	Shibaura	−
	TR-1	−
	Asakawa	−

Initial number of leptospires in the medium was 10^7 per ml.
+ Growth of $10^8\sim2\times10^8$ leptospires per ml after incubation for 4 to 6 days
− No growth after incubation for a month

cells per ml after incubation for 4 to 6 days. These could be serially subcultured. On the other hand, the remaining 3 strains, all belonging to *L. icterohaemorrhagiae*, did not grow. Cells of the strain Shibaura and TR-1 disappeared within 2 days after inoculation; Asakawa did not disappear so rapidly but did not grow.

The following experiment was done, using strain Shibaura, to obtain the mutant which would grow in SHENBERG's medium. This strain did not grow in SHENBERG's medium when it was inoculated with 10^7 cells per ml. Even when the number of inoculated leptospires was increased up to 2×10^9 per ml this strain did not grow. The number of leptospiral cells inoculated amounted approximately to 5×10^{10} in total, which failed to grow in SHENBERG's medium.

From these results we thought that the desired mutant could not be obtained by this

type of experimental procedure. Instead, an attempt was made first to obtain the mutant which would grow in the boiled serum medium, according to the procedure reported by YANAGAWA & WILSON, and to examine the possibility of the mutant growing in SHENBERG's medium. In the boiled serum medium, the strain Shibaura grew when inoculated with 10^7 or more organisms per ml. This culture could be successively subcultivated in the boiled serum medium with a smaller quantity of inoculum. The mutant, after 10 times subcultivation in the boiled serum medium, was decimally diluted with SHENBERG's medium, which was incubated at 30°C. The results are shown in table 2. The mutant which grew in the

TABLE 2 *Growth in SHENBERG's medium of the mutants (Shibaura) which grow in the boiled serum medium*

NUMBER OF INOCULATED CELLS per ml	GROWTH
1.6×10^7	+ 4 *
„ 10^6	+13
„ 10^5	+15
„ 10^4	+17
„ 10^3	•
„ 10^2	+23
„ 10	+27
1 or 2	+30

* +4 indicates that the growth (more than 10^8 cells per ml) was obtained after 4 days incubation.

• Could not be examined due to contamination

boiled serum medium was found to grow in SHENBERG's medium from a very small inoculum such as 1 or 2 cells per ml. This could be subcultivated serially in SHENBERG's medium. The maximal cell counts of the mutant in SHENBERG's medium were about 2×10^8 per ml. Thus, the strain Shibaura which originally did not grow in SHENBERG's medium became to grow in the same medium by using the mutant which could grow in boiled serum medium. Similarly, the mutant was also obtained from strain TR-1.

Properties of the mutant which grew in SHENBERG's medium were compared with those of the parent. For this purpose, 20th~25th subcultures of the mutant in SHENBERG's medium were used.

The maximal growth of both the parent and mutant in serum medium reached 3×10^8 cells per ml (on 4th day after inoculation, from 2×10^7 cells per ml), showing no difference between the parent and mutant.

The virulence of the parent and mutant were not different as indicated in table 3.

The antigenicity of each strain was compared by the reciprocal microscopic agglutination test (tab. 4) and immunodiffusion. Immunodiffusion of rabbit antisera to the antigens extracted with SDC from the parent and mutant is shown in figures 1 and 2. The results of these tests were that no difference of antigenicity was found between the parent and mutant.

TABLE 3 *Virulence of the parent and mutant*

NUMBER OF INOCULATED LEPTOSPIRES	PARENT	MUTANT*1
10^7	D 7*2	D 7
10^6	×	D 8
10^5	D 8	D 8
10^4	D 9	D 8
10^3	D 9	D 9

*1 22nd subculture in SHENBERG's medium
*2 D7 indicates death of guinea pig on the 7th day after inoculation.
× Death by accident

TABLE 4 *Cross microscopic agglutination test of the parent and mutant*

ANTIGEN	IMMUNE SERUM	
	Parent	Mutant
Mutant	30,000 *	100,000
Parent	30,000	100,000

* Reciprocal of microscopic agglutination titer

The colonies of the parent and mutant grown on the solidified medium were found to be distinctly different. The colonies of the parent ranged from 2 (compact) to 6 mm (diffuse), as shown in figure 3. The colonies of the mutant were uniform in diameter (1~1.5 mm) and compact, as shown in figure 4. Each colony of the parent and mutant was considered to be formed from a single leptospire, because the number of leptospires of the parent and mutant of strains Shibaura inoculated onto the solidified medium was equal to the number of the colonies.

DISCUSSION

YANAGAWA & WILSON classified leptospires by their ability to grow in the medium which had been boiled after the addition of 10% rabbit serum. The strains which did not grow and those which grew in the boiled serum medium were grouped respectively as types I and II. Furthermore, they selected the mutants of type I which grew in the boiled serum medium. STALHEIM & WILSON investigated the physiological differences between types I and II. The type I leptospires were rapidly lysed by monoolein and other lipids, while the type II leptospires were more resistant. They suggested that the limiting agent of the

boiled serum medium is a lytic action of free fatty acids released from the serum proteins by the boiling process. The role of the boiled serum medium is considered to be the selection in which leptospires resistant to fatty acids were selected from sensitive leptospires.

STALHEIM & WILSON and PHIBBS & VAN ESELTINE obtained the mutants which grew in their defined media, by means of using the mutant which can grow in the boiled serum medium. In the present report it was also found that the cultivation of leptospires in the boiled serum medium was found to be a useful procedure to obtain the mutant which grows in SHENBERG's medium.

It is interesting that the mutant preliminarily selected in the boiled serum medium could grow in SHENBERG's medium from a very small inoculum such as 1 or 2 leptospiral cells. This may indicate that no selection was made in SHENBERG's medium. Since the parent did not grow in SHENBERG's medium even from a very large inoculum we expected that further selection would be made in SHENBERG's medium. The result obtained was against our expectations. The reason is not clear.

YANAGAWA & WILSON reported that the mutant type I leptospire grown in boiled serum medium exhibit no changes in antigenicity revealed by the microscopic agglutination test. However, the size of colonies was markedly reduced in the mutant. STALHEIM & WILSON described that the mutant type I leptospires grown in their Tween synthetic medium were poorly agglutinated by a homologous antiserum and did not adequately protect guinea pigs against infection with the original strain. Later, STALHEIM reported that when the Tween synthetic medium was modified by increasing the amount of Tween 80 (polyoxyethylene sorbitan monooleate), the serological properties of leptospires, examined by the microscopic agglutination test, were not changed from the original strain, but their virulence was lost. In the present experiments, we found that the growth in the serum medium, the antigenicity (examined by the reciprocal microscopic agglutination test and immunodiffusion) and virulence were not different between the parent and mutant. Colonies of the mutant, however, were clearly distinguished from those of the parent.

Reviewing these results it seems that a phenotypic change of the leptospire would occur in succession to the mutation of leptospires during serial cultivation in the media different from the ordinary serum medium. And the appearance of the phenotypic change of leptospire may occur as follows: the decrease in the size of the colony may occur first, followed by the decrease of virulence, and then comes the change in antigenicity. However, this speculation should await further experiment.

References

1) Cox, C. D. (1955): *Proc. Soc. expl. Biol. Med.*, **90**, 610
2) Cox, C. D. & Larson, A. D. (1957): *J. Bact.*, **73**, 587
3) Fujikura, T. (1966): *Jap. J. Microbiol.*, **10**, 79
4) Johnson, R. C. & Gary, N. D. (1963): *J. Bact.*, **85**, 976
5) Murty. D. K. & Hanson, L. E. (1961): *Am. J. vet. Res.*, **22**, 274
6) Phibbs, P. V. & Van Eseltine, W. P. (1968): *J. inf. Dis.*, **118**, 197
7) Shenberg, E. (1967): *J. Bact.*, **93**, 1598,
8) Stalheim, O. H. V. (1966): *Ibid.*, **92**, 946
9) Stalheim, O. H. V. & Wilson, J. B. (1964): *Ibid.*, **88**, 48
10) Vogel, H. & Hutner, S. H. (1961): *J. gen. Microbiol.*, **26**, 223
11) Yanagawa, R. & Faine, S. (1966): *Nature, Lond.*, **211**, 823
12) Yanagawa, R., Shimizu, T., Hiramune, T. & Ishii, S. (1958): *Bull. natl. Inst. anim. Hlth.*, No. 36, 23
13) Yanagawa, R. & Wilson, J. B. (1962): *J. inf. Dis.*, **110**, 70

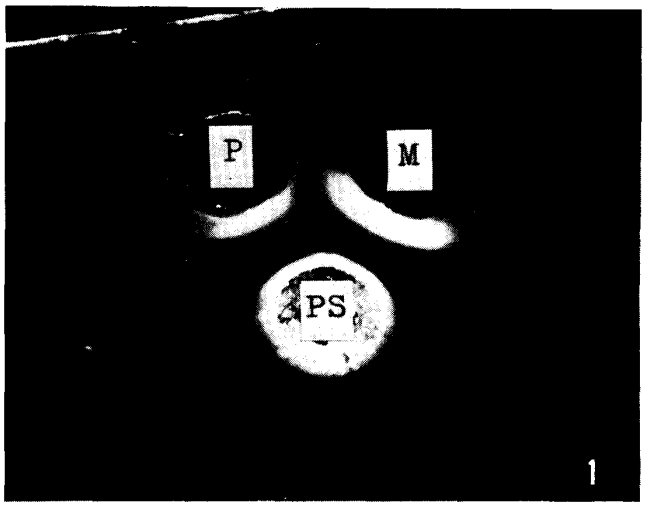
EXPLANATION OF PLATE

Figs. 1 & 2 Antigenicity of the parent and mutant strains of *L. icterohaemorrhagiae* Shibaura revealed by immunodiffusion

P antigen extracted by SDC from the parent strain
M antigen extracted by SDC from the mutant strain
PS concentrated immune serum of the parent
MS concentrated immune serum of the mutant

Fig. 3 Colonies of the parent (COX & LARSON medium, 12th day of incubation) × 2

Fig. 4 Colonies of the mutant (COX & LARSON medium, 12th day of incubation) × 2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M
PS
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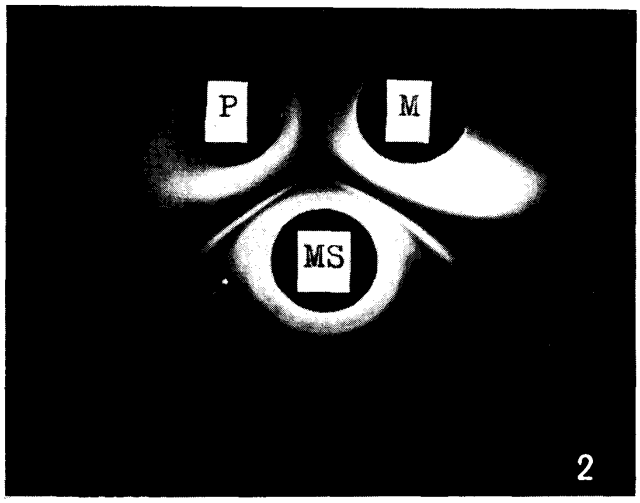
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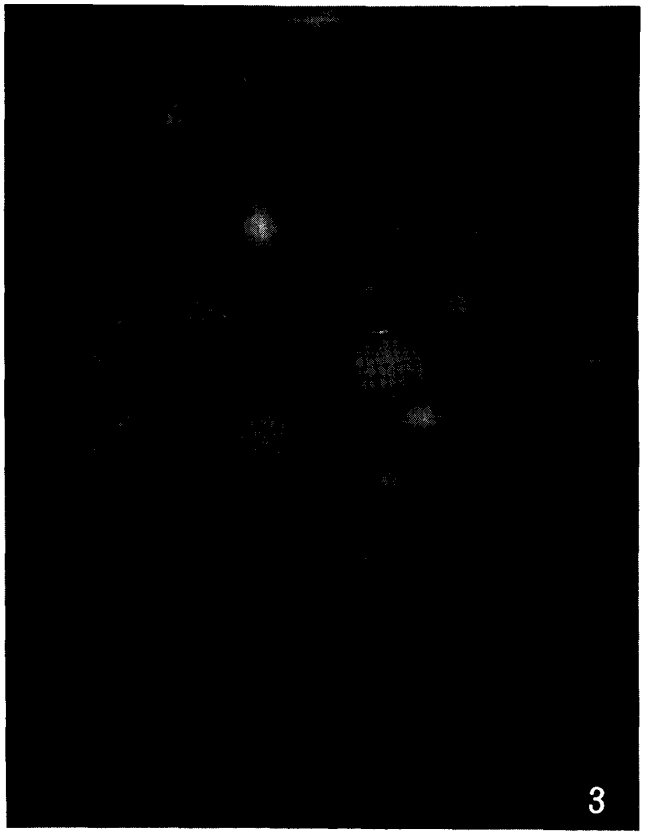
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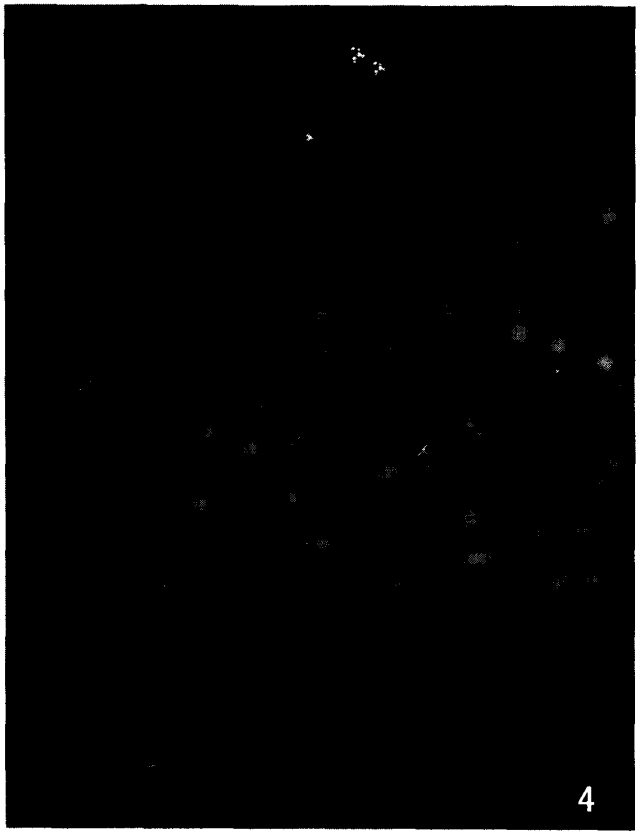
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