STUDIES OF LEPTOSPIRA HEBDOMADIS.

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Besides infectious jaundice (Weil's disease), there prevails in Japan an endemic disease which is symptomatically closely related to infectious jaundice but epidemiologically distinct: seven day fever (the *nanukayami*, of Fukuoka province), or autumn fever (*akiyami*, of Shizuoka province). Inada concluded from an epidemiological study of the two diseases that they were etiologically distinct. The isolation of *Leptospira icterohæmorrhagiæ* from infectious jaundice was accomplished by Inada and Ido in 1914, and in 1917 Ido, Ito, and Wani isolated a somewhat similar organism, *Leptospira hebdomadis*, from *nanukayami*. The biological properties of *Leptospira hebdomadis* were not determined, however, and in 1922 an expedition was sent to Shizuoka province for the study of *akiyami*. Numerous cases, in some years more than 100, occur in the region of the Oi River. The endemic season is practically limited to the period between August and October, the seasonal prevalence of *akiyami* being similar to that of *nanukayami* and quite different from that of infectious jaundice. The average morbidity is about 0.3 per cent. The infection occurs chiefly in persons engaged in cultivation of the soil. Most of the patients are under 20 years of age.

Thirty cases of *akiyami* were studied, clinically and bacteriologically. The date of the first case was September 6, of the last, October 13. Two types of *Leptospira hebdomadis* were isolated during this study. The characteristics of the two types will be discussed further on.

Isolation of the Leptospira.

In the isolation of the leptospira from *akiyami* patients we followed the original method of Inada and injected blood from the patients intraperitoneally into young guinea pigs (150 to 200 gm.). Some of the animals showed the typical

TABLE I.

Transmission from Patients to Guinea Pigs.

Case No.	Date of onset.	Day of disease on which blood was taken.	Direct culture from blood.	Amount of blood injected.	No. of guinea pigs inoculated.	Leptospira in blood of guinea pig.	Course after infection.	Autopsy. Jaundice.	Autopsy. Hemorrhage.	Autopsy. Leptospira in liver.	Results of subsequent passage.
	1922			*cc.*							
1	Sept. 6	3rd		2.0	2	+	D. in 8 days. " " 7 "	++ ++	+++ +++	+++ +++	Always +
2	" 12	2nd		2.0	2	−	Remained well.				
3	" 19	3rd		2.0	2	−	" "				
4	" 20	2nd		2.0	2	−	" "				
5	" 26	4th		2.0	1	+	Killed in 10 days.				− 2nd generation.
6	" 27	3rd		1.5	2	+ −	" " 7 " Remained well.	−	++	+	− 3rd "
7	" 28	4th		1.0	2	+ −	Killed in 10 days. Remained well.	−	++	+	− 2nd "
8	" 29	4th		1.5	2	− −	" " " "				
9	" 30	4th		1.5	2	+ +	D. in 9 days. " " 9 "	+++ ++	+++ +++	+++ +++	Always +

10	Sept. 30	4th		1.5	2	+ ?	D. in 8 days. Remained well.	−	+	+	
11	" 30	3rd		1.5	2	+ +	Killed in 7 days. Remained well.	−	++	+	− 2nd generation.
12	Oct. 1	3rd		1.5	2	+ +	" " D. in 9 days.	− −	++	+ +	
13	" 3	2nd		1.5	2	+ +	" " 9 " " " 12 "	− −	+ +	− −	 − 2nd "
14	" 6	3rd		1.5	1	?	" " 6 "	−	++	+	− 5th "
15	" 10	3rd	+	2.0	1	+	" " 6 "	−	+	+	− 3rd "
16	" 11	1st	+	1.5	2	+	Remained well. Lost.				
17	" 12	3rd		2.0	2	+	D. Lost.	−	++	+	− 2nd "
18	" 13	4th	+	1.5	2	+ +	D. in 9 days. " " 9 "	++ ++	+++ +++	++ ++	} Always +
19	" 13	3rd	+	1.5	2	 +	Lost. D. in 8 days.	 −	 ++	 +	 − 2nd generation.

In the tables D. indicates died; S., survived.

akiyami symptoms within 1 week. Daily examinations of the blood for the presence of the leptospira were made by means of the dark-field microscope. When the finding was positive, a sample of blood for cultivation was taken directly by heart puncture, without killing the animal. In the case of dead animals, liver emulsion was used for transmission. In some instances a few drops of the patient's blood were mixed with Ringer's solution-agar medium, and incubated at room temperature, or 30°C. Examinations were made after 7 to 10 days incubation.

Of nineteen cases which were examined by one or the other of these methods within 4 days after the beginning of the disease, the leptospira was found in fifteen. The details of the transmission experiments are recorded in Table I.

Several facts noted during the animal experiments deserve special attention. In some instances the animals show intense jaundice and hemorrhage, and the infection terminates fatally after a brief illness. In the liver emulsion of such animals a large number of the leptospiras are found. In some of the fatal instances only a slight jaundice may be observed; hemorrhage in the lungs is sometimes marked; the leptospiras in the liver are few in number, and the virulence of the strain diminishes gradually in two or three generations. In this respect the infection resembles very much that of experimental seven day fever (*nanukayami*). In other cases the animals infected with patients' blood show the presence of the leptospira in the blood but survive. These facts had already been noted by Kitamura, who, however, did not determine whether the mildness of the infection is due to variations in the virulence of the strains or to individual differences in the susceptibility of guinea pigs. From the results of our own repeated inoculation experiments, we regard such variations in the experimental disease as due to differences in virulence in the strains of spirochetes.

We obtained pure cultures of the spirochete in twelve of the nineteen cases; three of these (Nos. 1, 9, and 18) were of rather high virulence. The virulence of a given strain is not necessarily parallel to the clinical symptoms of the patient.

We have tentatively classified the *akiyami* leptospiras into two types, according to their virulence in animals, the more highly virulent A type, and the less virulent B type. Comparative immunological

studies have been carried out with *Leptospira icterohæmorrhagiæ* on the one hand and with *Leptospira hebdomadis* on the other.

Immunological Studies.

Pfeiffer's Phenomenon with the Sera of Convalescents.

Blood was withdrawn from sixteen convalescents, within 2 to 4 weeks after defervescence, and 1 cc. of the serum was mixed with 1 cc. of liver emulsion which

TABLE II.

Summary of Pfeiffer Reactions.

Strain of leptospira.	*Akiyami* convalescent sera.															Infectious jaundice.		Anti-*icterohæmorrhagiæ* serum (horse).
	S.M.	H.I.	S.K.	I.T.	M.T.	T.S.	G.Y.	R.T.	T.O.	T.T.	G.W.	J.O.	S.I.	G.K.	K.S.	I.A.		
	Day of disease on which serum was taken.																Case 1.	Case 2.	
	37	22	21	27	21	21	28	33		28	20	31							
Akiyami leptospira A type.																			
Strain 1 (Case 1).....	+	+	+	−		−			−		−	−	−				−	−	−
" 2 (" 9).....	+	+	+			−	−	−					−				−	−	−
" 3 (" 18).....	+	+	+	−	−	−			−	−							−	−	−
Akiyami leptospira B type.																			
Strain 1 (Case 6).....	−	+?	−		+	+			+		+	+	+	+	+	+	−		
" 2 (" 16).....			−						+	+		+		+					
" 3 (" 15).....	−						+								+				
Leptospira icterohæmorrhagiæ (Strain1).....	−	−	−			−	−										+	+	+
Leptospira hebdomadis (Strain 1).....	−				+							+		−					

showed by dark-field examination about 10 leptospiras per field. The mixtures were injected intraperitoneally into guinea pigs, and after 30 minutes, and again after 2 hours, liquid was withdrawn from the peritoneal cavity and examined microscopically for the presence of the organisms. The animals were observed for some time after this examination, and in the event of death autopsies were carried out and the pathological changes studied.

In the case of the B type, owing to the difficulty of obtaining suitable liver emulsions containing these organisms, we used Ringer's solution cultures of the respective strains in their most suitable stages of development. 1 cc. of culture was used in each Pfeiffer test. The examination of the peritoneal liquid was made 30 minutes, 2, 4, and 6 hours after injection.

The results of Pfeiffer tests are summarized in Table II.

A positive Pfeiffer reaction against the A type organism was obtained only with the sera of the patients from whom we had isolated the A type. Convalescent sera from other *akiyami* cases and from infectious jaundice had no effect whatever upon the A type organism. Conversely, we obtained positive reactions upon *Leptospira icterohæmorrhagiæ* with convalescent sera from infectious jaundice cases, but no effect at all with sera from *akiyami* cases.

The *akiyami* convalescent sera which reacted positively with the B type *akiyami* organism gave positive reactions also with *Leptospira hebdomadis*. The convalescent sera which reacted positively against the A type *akiyami* spirochete gave negative Pfeiffer reactions with the B type organism and with *Leptospira hebdomadis*.

As the table shows, the B type of *akiyami* leptospira seems to be identical with *Leptospira hebdomadis*, while the A type is serologically distinct from *Leptospira hebdomadis* as well as from *Leptospira icterohæmorrhagiæ*.

Preparation of Specific Immune Sera.

Monovalent immune sera were prepared in rabbits against each of the *akiyami* strains. We also used in the experiments the serum of a horse immunized against *Leptospira icterohæmorrhagiæ*, prepared in the Government Institute for Infectious Diseases of Tokyo Imperial University.

The immunization of rabbits was usually carried out according to Ōba's method. Sometimes Uhlenhuth's technique was used in the case of the A type leptospira and *Leptospira icterohæmorrhagiæ*. Occasionally intravenous injections were made of the leptospira cultures, but with rather less satisfactory results.

The technique of Ōba's method, namely the injection of culture intrahepatically, can be performed without laparotomy by using a rather long syringe needle. The culture should be injected very slowly. The rabbits injected gradually became emaciated; the development of jaundice depends on the virulence of injected spirochete antigen. The specific agglutinins appear in the serum within 4 to 6 weeks after injection.

When Uhlenhuth's method was used, we injected a large quantity

of liver emulsion intraperitoneally and intravenously at the same time. When the inoculation is successful, the animals show typical symptoms, and jaundice becomes very evident.

Agglutination Reactions.

The technique of agglutination reaction is far more difficult with spirochetes than with most bacteria. Ōba in 1921 published a simpli-

TABLE III.

Agglutination Reactions with Immune Serum against A Type Leptospira (Strain 3).

Test-tube No.	Mixture of			Final dilution of serum.	Tested against		
	0.4 per cent agar.	+ anti-*hebdomadis* A immune serum.			*Akiyami* leptospira A type (Strain 1).	*Akiyami* leptospira A type (Strain 3).	*Leptospira icterohemorrhagiæ* (Strain 1).
	cc.		*cc.*				
1	2.0	Undiluted.	0.5	1:5	+	+	−
2	2.0	1:2 dilution in normal rabbit serum.	0.5	1:10	+	+	−
3	2.0	1:4 " " " " "	0.5	1:20	+	+	−
4	2.0	1:8 " " " " "	0.5	1:40	+	+	−
5	2.0	1:16 " " " " "	0.5	1:80	+	+	−
6	2.0	1:32 " " " " "	0.5	1:160	+	+	−
7	2.0	1:64 " " " " "	0.5	1:320	+	+	−
8	2.0	1:128 " " " " "	0.5	1:640	+	+	−
9	2.0	1:256 " " " " "	0.5	1:1280	−	−	−
10	2.0	Normal rabbit serum.	0.5	Control.	−	−	−

+, agglutination and degeneration; −, normal growth, no effect.

fication of his original technique of agglutinin determination *in vitro* for application to *Leptospira icterohæmorrhagiæ*. The details are as follows:

2 cc. of sterile 0.3 per cent Ringer's solution-agar is distributed into a series of test-tubes, which are then resterilized. 1 cc. of rabbit immune serum is taken up into a syringe with a long needle, 0.5 cc. of which is put into the first tube; 0.5 cc. of normal rabbit serum is taken up into the syringe to dilute the immune serum 1:2; 0.5 cc. of twofold diluted immune serum is delivered into the second test-tube; this procedure is repeated throughout the series of tubes. The serum is

mixed with the melted agar. Before coagulation 1 or 2 drops of spirochete-containing material is added to each tube. The tubes are placed at 37°C. After 3 or 4 days incubation, examinations are made by means of dark-ground illumination.

In the case of positive reaction the multiplied organisms lose their motility and are agglutinated into masses. In the strongly positive instances almost no individual spirochetes can be recognized in the conglomerate masses, which have a

TABLE IV.

Agglutination with A Type Immune Sera.

Strain of leptospira.	Serum 1 (titer 1:320).						Serum 3 (titer 1:640).						
	Dilution of serum.												
	1:10	1:40	1:80	1:160	1:320	Control.	1:10	1:20	1:80	1:160	1:320	1:640	Control.
A type leptospira.													
Strain 1 (Case 1)	+	+	+	+	+	−	+	+	+	+	+	+	−
" 2 (" 9)							+	+	+	+	+	+	−
" 3 (" 18)	+	+	+	+	+	−	+	+	+	+	+	+	−
B type leptospira.													
Strain 1 (Case 6)	−		−		−	−	−	−	−	−	−	−	−
" 2 (" 16)	−	−	−	−		−							
" 3 (" 15)	−	−	−			−							
" 4 (" 5)		−		−		−							
Leptospira icterohæmorrhagiæ.													
Strain 1	−	−	−	−	−	−	−	−	−	−	−	−	−
" 2	−	−	−	−	−	−							
" 3		−		−	−	−							
Leptospira hebdomadis.													
Strain 1	−	−	−	−	−	−							
" 2							−	−	−	−	−	−	−

granular appearance in the center. In a weakly positive reaction, the multiplied organisms show slight motility, especially in the peripheral part of the agglomerates. In all cases microscopic examination is necessary to avoid any possible errors in the examination.

The results of the agglutination tests of all the leptospira strains against monovalent sera prepared with the A type (two strains), the

B type (three strains), *Leptospira icterohæmorrhagiæ* (one strain), and *Leptospira hebdomadis* (one strain) are summarized in Tables III to VI.

It may be safely concluded from the results shown in these tables that the leptospira can be serologically identified by the method used. All four strains of *Leptospira icterohæmorrhagiæ* behave serologically

TABLE V.

Agglutination with B Type Immune Sera.

Strain of leptospira.	Serum 1 (titer 1:160).					Serum 3 (titer 1:320).					
	Dilution of serum.										
	1:10	1:40	1:80	1:160	Control.	1:10	1:20	1:40	1:80	1:160	1:320	Control.
A type leptospira.												
Strain 1	−	−	−	−	−							
" 3	−	−	−	−	−							
B type leptospira.												
Strain 1	+	+	+	+	−	+	+	+	+	+	+	−
" 2						+	+	+	+			−
" 3	+	+	+	+	−	+	+	+	+	+	+	−
" 4						+	+	+	+			−
" 5	+	+	+	+	−	+	+	+	+			−
Leptospira icterohæmorrhagiæ.												
Strain 1	−	−	−	−	−							
" 2	−	−	−	−	−							
" 3	−	−	−		−							
Leptospira hebdomadis.												
Strain 1	+	+	+	+	−	+	+	+	+	+	+	−

exactly in the same manner; they can be readily differentiated from the three strains of *akiyami* A type organism on the one hand, and from the *akiyami* B type and *Leptospira hebdomadis* on the other.

Protective Property of the Immune Sera.

The immune rabbit sera used in this part of the work were prepared by Uhlenhuth's method. An immune horse serum against *Leptospira icterohæmorrhagiæ,*

without preservative, was also utilized. The sera were diluted 1:10, 1:20, 1:50, and 1:100, and 1.0 cc. of a given immune serum or its dilution was mixed with 1.0 cc. of liver emulsion of infected guinea pigs, and the whole amount of the mixture was injected into each guinea pig. In case of death, liver emulsion was examined by means of the dark-field microscope.

TABLE VI.

Agglutination with Immune Sera against Leptospira icterohæmorrhagiæ and Leptospira hebdomadis.

Strain of leptospira.	Anti-*icterohæmorrhagiæ* immune serum (titer 1:320).							Anti-*hebdomadis* immune serum (titer 1:160).					
	Dilution of serum.												
	1:10	1:20	1:40	1:80	1:160	1:320	Control.	1:10	1:20	1:40	1:80	1:160	Control.
A type leptospira.													
Strain 1	−	−					−	−	−	−	−	−	−
" 3	−	−	−				−	−			−		−
B type leptospira.													
Strain 1		−		−	−	−	−	+	+	+	+	+	−
" 2	−	−	−	−	−	−	−						
" 3	−	−	−	−	−	−	−	+	+	+	+	+	−
" 5	−	−	−	−			−						
Leptospira icterohæmorrhagiæ.													
Strain 1	+	+	+	+	+	+	−						
" 2	+	+	+	+		+	−	−	−	−	−	−	−
" 3	+	+	+			+	−						
" 4	+		+	+	+		−						
Leptospira hebdomadis.													
Strain 1	−	−	−	−	−	−	−	+	+	+	+	+	−

The results of one series of the present experiment are shown in Table VII.

The anti-*icterohæmorrhagiæ* immune sera protected guinea pigs against infection with *Leptospira icterohæmorrhagiæ* when given in amounts as small as 0.001 cc., but not against infection with the *akiyami* A type leptospira, even in 1 cc. The *akiyami* A type immune serum protects guinea pigs against the A type infection in 0.01 cc. but not against infection with *Leptospira icterohæmorrhagiæ* in 0.1 cc.

TABLE VII.

Protective Property of Immune Sera.

Anti-*icterohæmorrhagiæ* immune rabbit serum.														Rabbit immune serum against *akiyami* leptospira A type (Strain 3).											
+ *akiyami* leptospira A type (Strain 1).							+ *Leptospira icterohæmorrhagiæ* (Strain 1).							+ *akiyami* leptospira A type (Strain 1).						+ *Leptospira icterohæmorrhagiæ* (Strain 1).					
Amount of immune serum.*	Infective liver emulsion.	Body weight of guinea pig.	Course.	Autopsy.			Amount of immune serum.*	Infective liver emulsion.	Body weight of guinea pig.	Course.	Autopsy.			Amount of immune serum.*	Infective liver emulsion.	Body weight of guinea pig.	Course.	Autopsy.			Amount of immune serum.*	Infective liver emulsion.	Body weight of guinea pig.	Course.	Autopsy.		
				Icterus.	Hemorrhage.	Leptospira in liver.					Icterus.	Hemorrhage.	Leptospira in liver.					Icterus.	Hemorrhage.	Leptospira in liver.					Icterus.	Hemorrhage.	Leptospira in liver.
cc.	*cc.*	*gm.*					*cc.*	*cc.*	*gm.*					*cc.*	*cc.*	*gm.*					*cc.*	*cc.*	*gm.*				
1.0	1.0	170	D. in 7 days.	+	++	+								1.0	1.0	175	S.				1.0	1.0	172	D. in 10 days.	−	−	−
0.1	1.0	162	D. in 6 days.	++	++	++	0.1	1.0	190	S.				0.1	1.0	180	"				0.1	1.0	195	D. in 7 days.	+++	+++	++
0.05	1.0	200	D. in 6 days.	+++	+++	++	0.05	1.0	200	"				0.05	1.0	185	"				0.05	1.0	195	D. in 7 days.	++	++	+
0.02	1.0	180	D. in 6 days.	+++	+++	++	0.02	1.0	204	"				0.02	1.0	180	"				0.02	1.0	195	D. in 3 days.			
0.01	1.0	185	D. in 5 days.	++	+++	++	0.01	1.0	204	"				0.01	1.0	185	"				0.01	1.0	207	D. in 7 days.	+++	+++	++
0	1.0	215	D. in 6 days.	+++	+++	++	0	1.0	207	D. in 7 days.	+++	++	+++	0	1.0	207	D. in 6 days.				0	1.0	205	D. in 6 days.	+++	+++	++

* Ringer's solution was used to bring the amounts of immune serum to a uniform volume of 1 cc.

TABLE VIII.

Protective Property of Immune Sera.

Anti-*icterohæmorrhagiæ* immune horse serum.															Rabbit immune serum against *akiyami* leptospira A type (Strain 3).											
+ *akiyami* leptospira A type (Strain 1).							+ *Leptospira icterohæmorrhagiæ* (Strain 1).								+ *akiyami* leptospira A type (Strain 1).							+ *Leptospira icterohæmorrhagiæ* (Strain 1).						
Amount of immune serum.	Infective liver emulsion.	Body weight of guinea pig.	Course.	Autopsy.			Amount of immune serum.	Infective liver emulsion.	Body weight of guinea pig.	Course.	Autopsy.			Amount of immune serum.	Infective liver emulsion.	Body weight of guinea pig.	Course.	Autopsy.			Amount of immune serum.	Infective liver emulsion.	Body weight of guinea pig.	Course.	Autopsy.		
				Icterus.	Hemorrhage.	Leptospira in liver.					Icterus.	Hemorrhage.	Leptospira in liver.					Icterus.	Hemorrhage.	Leptospira in liver.					Icterus.	Hemorrhage.	Leptospira in liver.
cc.	*cc.*	*gm.*					*cc.*	*cc.*	*gm.*						*cc.*	*cc.*	*gm.*					*cc.*	*cc.*	*gm.*				
1.0	1.0	180	D. in 5 days.	+++	−	+									0.02	1.0	150	S.				1.0	1.0	145	D. in 8 days.	++	+	+
1.0	1.0	200	D. in 2 days.	−	−	−	0.02	1.0	155	S.					0.02	1.0	155	"				1.0	1.0	160	D. in 11 days.	−	−	−
0.5	1.0	200	D. in 5 days.	+++	−	+	0.01	1.0	205	"					0.01	1.0	145	"				0.5	1.0	202	S.			
0.5	1.0	160	D. in 5 days.	+++	+	++	0.01	1.0	155	D. in 19 days.	−	−	−		0.01	1.0	155	D. in 8 days.	−	−	−	0.5	1.0	180	"			
0.2	1.0	204	D. in 7 days.	+++	+	++	0.005	1.0	160	D. in 16 days.	−	−	−		0.005	1.0	160	D. in 11 days.	−	+	+	0.2	1.0	220	D. in 8 days.	+++	+++	++

0.2	1.0	208	D. in 5 days.	+++	+	++	0.005	1.0	140	D. in 16 days.	–	–	–	0.005	1.0	215	S.				0.2	1.0	170	D. in 7 days.	++	+++	++
0.1	1.0	207	D. in 7 days.	+++	++	++	0.002	1.0	215	D. in 11 days.	++	+++	++	0.002	1.0	175	D. in 8 days.	–	+	+	0.1	1.0	190	D. in 8 days.	++	++	++
0.1	1.0	170	D. in 6 days.	+++	++	++								0.002	1.0	152	D. in 11 days.	+++	+++	++	0.1	1.0	152	D. in 8 days.	+++	+++	++
0	1.0	160	D. in 6 days.	+++	+++	+++	0	1.0	210	D. in 6 days.	++	++	++	0	1.0	270	D. in 6 days.	++	+++	++	0	1.0	200	D. in 7 days.	++	+++	++

The guinea pig given 1 cc. of this serum died on the 10th day. The autopsy findings were negative and the cause of death unknown.

In similar experiments (Table VIII) in which relatively smaller amounts of homologous and relatively larger amounts of heterologous sera were used, we obtained nearly the same results as before; that is, 0.005 cc. (1.0 cc. of 1:200 dilution) of anti-*icterohæmorrhagiæ* immune serum protected against the homologous infection, but even 1 cc. did not protect against the A type infection. The A type immune serum always protected in 0.01 cc., and often in 0.005 cc., but the protection was imperfect against heterologous infection even with 1 cc.; (with 0.2 cc. of the immune serum no sign of any positive influence is noticeable).

This experiment brought out a very interesting point with regard to the relation of *Leptospira icterohæmorrhagiæ* and the A type. Though amounts of 1 or 0.5 cc. of anti-*icterohæmorrhagiæ* immune serum do not protect against the A type infection, we did not see at autopsy any marked hemorrhage in the various organs and could find only a few leptospiras in the liver emulsion, while the degree of jaundice was very intense. These findings may possibly indicate a beneficial effect of the immune bodies of the serum, which diminish the action of the infecting organisms, though unable to protect against infection.

The same experiments were carried out six times in succession and always with nearly the same results. We conceive that there is some slight mutual protective influence of the immune sera of *icterohæmorrhagiæ* and the A type when large amounts are used, while remarkable differences in the serological behavior of the organisms are evident when the sera are used in higher dilutions.

Active Immunization.

It has been found that guinea pigs which are susceptible to *Leptospira icterohæmorrhagiæ* can be immunized with dead cultures of the organism. Hence it was of special interest to inoculate guinea pigs with the other types of leptospira in the same way and to test their immunity with homologous as well as heterologous strains. The antigens were prepared as follows:

TABLE IX.

Infection Tests of Guinea Pigs after Active Immunization.

	Antigen.																					
	Leptospira icterohæmorrhagiæ.							*Akiyami* leptospira A type.					*Akiyama* leptospira B type.				*Leptospira hebdomadis.*				Control.	
	Guinea pig No.																					
	1	2	3	4	5	6	7	1	2	3	4	5	1	2	3	4	1	2	3	4	1	2
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*		
Preparatory injections.																						
Apr. 11.....	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5		
" 18.....	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5		
" 25.....	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0		
Subsequent test with infective liver emulsion. May 10	*Leptospira icterohæmorrhagiæ.*				*Akiyami* leptospira A type.			*Leptospira icterohæmorrhagiæ.*			*Akiyami* lepto-spira A type.		*Leptospira ictero-hæmorrhagiæ.*		*Akiyami* leptospira A type.		*Leptospira ictero-hæmorrhagiæ.*		*Akiyami* leptospira A type.		*Leptospira icterohæ-morrhagiæ.*	*Akiyami* leptospira A type.
	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.
Course after injection.....	S.	S.	S.	D. in 6 days.	D. in 5 days.	D. in 4 days.	D. in 6 days.	D. in 6 days.	D. in 5 days.	D. in 7 days.	S.	S.	D. in 5 days.	D. in 5 days.	D. in 6 days.	D. in 5 days.	D. in 5 days.	D. in 7 days.	D. in 6 days.	D. in 6 days.	D. in 6 days.	D. in 5 days.
Autopsy.....				+	+	+	+	+	+	+			+	+	+	+	+	+	+	+	+	+

TABLE X.

Infection Tests of Guinea Pigs after Active Immunization.

	Antigen.															
	Leptospira icterohæmorrhagiæ.							*Akiyami* leptospira A type.					Control.			
	Guinea pig No.															
	1	2	3	4	5	6	7	1	2	3	4	5	1	2	3	4
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*				
Preparatory injections.																
June 14	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5				
" 20	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0				
" 26	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0				
Subsequent injection with living culture, July 7	*Leptospira icterohæmorrhagiæ.*				*Akiyami* leptospira A type.			*Leptospira icterohæmorrhagiæ.*		*Akiyami* leptospira A type.			*Leptospira icterohæmorrhagiæ.*	*Akiyama* leptospira A type.		
	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.		
Course after injection	S.	S.	S.	S.	D. in 7 days.	D. in 11 days.	S.	D. in 8 days.	S.	S.	S.	S.	D. in 8 days.	D. in 7 days.		
Autopsy					+	+		+					+	+		

Final test with infective liver emulsion. July 22	*Leptospira icterohæmorrhagiæ.*		*Akiyami* leptospira A type.							*Leptospira icterohæmorrhagiæ.*		*Akiyami* leptospira A type.			*Leptospira icterohæmorrhagiæ.*	*Akiyami* leptospira A type.
	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.						1.0 cc.	1.0 cc.	1.0 cc.			1.0 cc.	1.0 cc.
Course after injection.....	S.	S.	D. in 9 days.	D. in 16 days.						D. in 6 days.	S.	S.			D. in 7 days.	D. in 6 days.
Autopsy.....			+	+						+					+	+

Each strain was cultivated in two media, 0.4 per cent Ringer's solution-agar with normal rabbit serum, and Ringer's solution media with normal rabbit serum. At the maximum stage of growth the cultures grown on the agar medium were mixed with the other cultures in proportions of 2:1 and heated for 30 minutes at 57°C. The guinea pigs were given three intraperitoneal injections at 6 to 7 day intervals. 2 weeks after the last injection the degree of active immunity was tested by infection.

Table IX is a summary of the results of immunity tests in one series of such experiments.

It is evident that serological differences exist between *Leptospira icterohæmorrhagiæ* and the *akiyami* A type leptospira.

In another series of guinea pigs we injected living culture following the three injections of vaccine and tested the immunity by subsequent injection of liver emulsion (Table X).

We lost no guinea pigs by using a homologous living strain for the fourth dose, but with one exception all the animals died which received a heterologous living strain on the fourth injection. In guinea pigs actively immunized with a dose of liver emulsion the result was not so precise as before. Nevertheless, we can conclude that rather sharp serological differences exist between *Leptospira icterohæmorrhagiæ* and the A type of leptospira.

Another series of experiments was designed to determine whether or not actively immunized guinea pigs would be able to withstand infection with a heterologous strain.

The preparatory injections were made subcutaneously at 4 day intervals; 1.5 cc. of the vaccine was given at the first injection, 2 cc. at each of three succeeding injections; there followed an injection of 1.5 cc. of living old culture, one of 2.0 cc. of a fresh culture, and, for the seventh and last injection, 1.0 cc. intraperitoneally of liver emulsion of infected guinea pig. The subsequent infection test was performed on the 20th day after the last injection, 1.5 cc. of liver emulsion of a guinea pig infected with heterologous strain being given intraperitoneally. Among the six guinea pigs thus tested, two showed no sign of infection, three developed typical symptoms and died, and the other died of intercurrent infection.

Infection tests of this kind with the B type leptospira and *Leptospira hebdomadis* are impracticable owing to the lower virulence of these organisms and the growth of the guinea pigs to insusceptible adults during the course of the immunization. In these instances Pfeiffer's phenomenon was used as a test of immunity. 3 to 4 cc.

of culture in Ringer's solution of each organism was injected intraperitoneally into actively immunized guinea pigs, and samples of the peritoneal fluid were withdrawn several times at intervals of 30 minutes, 2, 4, and 6 hours, and examined microscopically for the fate of the injected leptospira (Table XI).

The B type of *akiyami* organism and *Leptospira hebdomadis* underwent lysis *in vivo* in the guinea pigs immunized with reciprocal heterologous strains, while neither showed any sign of lysis in guinea pigs immunized against A type or *Leptospira icterohæmorrhagiæ*.

TABLE XI.

Fate of B Type Leptospira and of Leptospira hebdomadis in Peritoneal Cavity of Actively Immunized Guinea Pigs.

Immunized against	Culture injected.	Amount.	Result.
		cc.	
B type leptospira.	B type leptospira (Strain 1).	3	+
A " "	" " " (" 1).	3	−
Leptospira hebdomadis.	" " " (" 1).	3	+
" *icterohæmorrhagiæ.*	" " " (" 1).	3	−
B type leptospira.	*Leptospira hebdomadis.*	4	+
A " "	" "	4	−
Leptospira hebdomadis.	" "	4	+
" *icterohæmorrhagiæ.*	" "	4	−?*
Control (not immunized).	B type leptospira.	3	−
" " "	*Leptospira hebdomadis.*	4	−

* Occasional non-motile leptospiras on first three examinations; none after 6 hours.

DISCUSSION.

As already stated, Inada and Ido had demonstrated in 1914 that *Leptospira icterohæmorrhagiæ* is the causal agent of infectious jaundice in Japan. Uhlenhuth, in 1916, showed independently that Weil's disease of Germany is caused by the same organism, and other observers (Stokes and Ryle, Martin and Pettit, Monti, and others) demonstrated it in cases of infectious jaundice occurring in Europe during the war. Noguchi (1918) isolated two strains of *Leptospira icterohæmorrhagiæ* from wild rats in America and made a thorough study of the biological relations existing between the Japanese, European, and American strains, which proved to be identical. Kaneko, in 1921, also reported that the European and Japanese strains are serologically identical. In 1918, Ido, Ito, and Wani found a

similar organism to be the etiological agent of *nanukayami*, or seven day fever, a disease which might be considered symptomatically as atypical infectious jaundice, but which Inada, in 1910, had concluded was etiologically distinct. This disease is variously known in different parts of Japan as *akiyami* (Shizuoka province), *akinetsu* (Kochi province), and *sakushyunetsu* (Okayama province). The causative organism (*Leptospira hebdomadis*) can be differentiated from *Leptospira icterohæmorrhagiæ* by serological reactions[1] as shown by Ido, and confirmed by Kaneko, and by Ōba, and their etiological identity is generally accepted.

Kitamura, in 1918, reported that the spiral organism which he found in cases of *akiyami* disease is very difficult to differentiate from *Leptospira hebdomadis* and *Leptospira icterohæmorrhagiæ*. Some of the strains which he isolated showed a virulence approaching that of *Leptospira icterohæmorrhagiæ*, others resembled *Leptospira hebdomadis* in this respect. He did not attempt serological identification.

Leptospira icteroides, first discovered by Noguchi, in 1919, in yellow fever cases in Guayaquil, is morphologically practically identical but serologically distinct from *Leptospira icterohæmorrhagiæ*. The behavior of *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ* in Pfeiffer's phenomenon and in agglutination and complement fixation reactions is distinctly different; in active immunization tests the differentiation is less marked. *Leptospira icteroides* is therefore biologically closely related to *Leptospira icterohæmorrhagiæ*, but serologically distinct.

We have found, as originally stated by Kitamura, that there are two types of leptospira causing *akiyami*, types differing in their virulence for guinea pigs. By means of the Pfeiffer reaction with convalescent sera, we have shown that the two strains are serologically distinct and have called the more virulent the A type, and the other the B type. Further serological study showed the B type to be identical with *Leptospira hebdomadis*, of *nanukayami*, while the A type is distinct from both *Leptospira hebdomadis* and *Leptospira icterohæmorrhagiæ*. The serological distinctions among these organisms were marked in Pfeiffer tests and agglutination reactions, but rather slight in active immunization tests. We regard the difference between our A type leptospira and *Leptospira icterohæmorrhagiæ* as probably of about the same degree as that between *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ*.

[1] Noguchi has recently shown that *Leptospira hebdomadis* can be morphologically differentiated from *Leptospira icterohæmorrhagiæ*, when grown under identical conditions, by its greater length and by its straighter body, the ends being hooked only rarely in living specimens.

The Relation of the Akiyami Leptospira in Rats.

That wild rats are the carriers in nature of *Leptospira icterohæmorrhagiæ* and field mice of *Leptospira hebdomadis* has been shown by Inada and Ido. We obtained from the endemic district in which our work was done five wild rats (*Mus decumanus*) and ten field mice (*Microtus montebelli*). The examination of the rats was repeatedly negative, both in urine and kidney tissue, but in the urine of one field

TABLE XII.

Agglutination Reactions with Immune Rabbit Serum Prepared with Leptospira from the Field Mouse.

Strain of leptospira.	Dilution of serum.						Control.
	1:5	1:10	1:20	1:80	1:160	1:320	
Leptospira from the field mouse	+	+	+	+	+	+	−
Akiyami leptospira A type.							
Strain 1	+	+	+	+	+	+	−
" 3	+	+	+	+			−
Akiyami leptospira B type.							
Strain 2	−	−	−	−	−	−	−
" 3	−	−	−	−			−
Leptospira icterohæmorrhagiæ.							
Strain 2	−	−	−	−			−
" 4	−	−	−	−			−
Leptospira hebdomadis.							
Strain 1	−	−	−	−			−

mouse a leptospira was found. The urine was injected intraperitoneally into guinea pigs, which died on the 8th day after injection. Many leptospiras were found in an emulsion of the liver which was inoculated into other guinea pigs. The strain was carried to further generations by inoculation of kidney suspension of the original mouse, and the inoculated animals died on the 17th day after injection. The virulence of this strain is so high that even after artificial cultivation for eight generations it continues to kill guinea pigs of over 450 gm.,

and by immunization of rabbits with the strain we obtained an immune serum of 1:320 titer. This serum was tested against *Leptospira icterohæmorrhagiæ*, *Leptospira hebdomadis* A, and *Leptospira hebdomadis* B, with the results shown in Table XII.

The agglutination reactions, and subsequent protection tests, showed that the field mouse strain is identical with the A type of *Leptospira hebdomadis*. Although only one mouse of the ten was found to harbor the strain, its transmission by field mice (*Microtus montebelli*) is indicated, and further search for the leptospira in field mice in endemic districts is desirable in connection with the epidemiological study of *akiyami*.

SUMMARY.

From the results of etiological study of the disease known as *akiyami* which prevails in the Shida district of Shizuoka province, we conclude that:

1. *Akiyami* is an infectious disease caused by a leptospira.

2. The leptospira causing *akiyami* is very difficult to differentiate morphologically from *Leptospira icterohæmorrhagiæ* and *Leptospira hebdomadis*.

3. The strains of leptospira isolated from cases of *akiyami* are of two types. One type, isolated from three of the sixteen cases, is highly virulent for guinea pigs and is serologically distinct from *Leptospira icterohæmorrhagiæ* and *Leptospira hebdomadis*; we have called this the A type. The type obtained from the other cases is less virulent for guinea pigs and is serologically identical with *Leptospira hebdomadis*; we have called this the B type.

4. The field mouse (*Microtus montebelli*), which harbors *Leptospira hebdomadis*, has also been found to harbor the A type of *akiyami*.

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