

IMMUNOLOGICAL STUDIES WITH A STRAIN OF LEPTO-
SPIRA ISOLATED FROM A CASE OF YELLOW
FEVER IN MERIDA, YUCATAN.

By HIDEYO NOGUCHI, M.D., AND I. J. KLIGLER, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, April 3, 1920.)

*Morphology, Cultural Properties, and Virulence of the Strain of
Leptospira Isolated in Merida.*

The morphological features and cultural properties of the strain of *Leptospira icteroides* isolated in Merida are similar to those of the Guayaquil strains.¹ With respect to pathogenicity and virulence we obtained the following data.

Guinea Pig 112 was inoculated on January 4, 1920, with the mixed emulsions of liver and kidney from Guinea Pig 43, which had typical symptoms as a result of inoculation of a culture made with blood drawn from Case 2 on December 19, 1919. No. 112 came down with typical symptoms on the 8th day and was killed for transfer on January 12. The liver and kidney were emulsified together with 0.9 per cent saline solution, about 1 gm. of organ material to 10 cc. of saline solution. The emulsion was allowed to stand several minutes until the supernatant fluid was free from coarse tissue particles. The clear portion, which under the dark-field microscope showed a few leptospiras in every field, was used to determine the minimum lethal dose for guinea pigs. The procedure consisted in inoculating intraperitoneally graduated amounts of the emulsion into as many guinea pigs as the number of dilutions required, each amount being contained in a uniform volume of 1 cc. of 0.9 per cent saline solution. The results of the experiment are recorded in Table I.

The period of incubation varied from 3 to 7½ days, and death occurred 7 to 10 days after the time of inoculation. The duration

¹ Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.

of illness, calculated from the beginning of fever until death, varied from $2\frac{1}{2}$ to $4\frac{1}{2}$ days. We did not succeed in finding the minimum lethal dose, which apparently lies below 0.0001 cc., the smallest quantity of emulsion employed in the present experiment.

The Merida strain showed a marked pathogenicity for young pups, 6 weeks old, when given intraperitoneally.²

TABLE I.

Determination of the Minimum Lethal Dose of the Merida Strain.

Guinea pig No.	Amount of organ emulsion.	Length of incubation.	Time of death.
	<i>cc.</i>	<i>days</i>	<i>days</i>
163	0.5	3	7
164	0.2	$3\frac{1}{2}$	8
165	0.1	$3\frac{1}{2}$	7
166	0.01	5	8
167	0.001	$7\frac{1}{2}$	10
168	0.0001	5	9

Dog 1.—Jan. 14, 1920. Inoculated with 4 cc. of the kidney emulsion from Guinea Pig 102 (second passage in guinea pigs) intraperitoneally. The temperature was 39.6°C. on the 4th day, falling rapidly to 38° the next morning. In the afternoon of the 7th day the animal was found near collapse (37°) and was killed for examination. Jaundice had been noticed in the conjunctivæ on the 6th day, and there was general jaundice on the 7th day. Albuminuria began on the 5th day and increased until the animal was killed.

Autopsy.—General icterus; marked congestion of the lungs and other organs. Hemorrhagic foci were found in small number in the lungs and intestinal mucosa. The kidneys were congested and degenerated. The liver was mottled with yellowish patches. No subcutaneous or intramuscular hemorrhages were found. The serous membranes were free from hemorrhage.

Dog 2.—This animal received the same material as Dog 1 at the same time. The temperature curve was somewhat more irregular, 39°C. on the 2nd and 3rd days, normal on the 4th day, and 39.6° on the 5th day. The decline was very rapid, 37.5° a.m. and 36.2° p.m. on the 6th day. The animal died during the night. Albuminuria was present on the 4th day. Jaundice was noticeable on the 5th day and had definitely increased during the 6th day.

Autopsy.—General jaundice; liver and other organs yellowish. Few small hemorrhagic foci in the lungs. The stomach contained some digested blood,

² Villamil Mendoza, M., Apuntes acerca de la fiebre amarilla, Thesis, Merida, 1920.

and the mucosa of the intestines was profusely hemorrhagic. The kidneys were congested and degenerated. Subcutaneous tissues, muscles, and serosa not hemorrhagic.

Identification of the Merida Strain and the Therapeutic Value of Anti-icteroides Serum against This Strain.

The identity of the Merida strain of the leptospira was established by means of a series of experiments in which varying quantities of anti-*icteroides* serum were injected, together with 1 cc. of an emulsion of the liver and kidney from Guinea Pig 43, into several normal

TABLE II.

Identification of the Merida Strain and the Therapeutic Value of Anti-icteroides Serum against This Strain.

Guinea pig No.	Amount of serum.	Result.	Lesions.
	cc.		
109	0 (control).	Killed after 6 days.	Typical.
110	0 (").	Died " 6 "	"
111	0 (").	Killed " 4 "	" lung lesions.
112	0.1	Survived.	
113	0.01	"	
114	0.001	"	
115	0.0001	"	

guinea pigs. The anti-*icteroides* serum had been prepared in a horse by repeated injections of different strains of *Leptospira icteroides* from Guayaquil cases. The results are recorded in Table II.

The outcome of this experiment made it evident that the Merida strain belongs to the same group as the Guayaquil strain.

Polyvalent anti-*icteroides* immune serum prepared in the horse or monovalent anti-*icteroides* immune serum prepared in the rabbit had a definite devitalizing action upon the Merida strain, while antisera similarly prepared with strains of *icterohæmorrhagiæ* had no perceptible effect upon the Merida strain.

Serotherapeutic Experiments.

The purpose of the next series of experiments was to ascertain whether or not the same immune serum possessed a therapeutic value in infection with the Merida strain such as had been demonstrated in the case of experimental infection in guinea pigs with the Guayaquil strains.³ Thirty-one guinea pigs were inoculated at the same time with 0.5 cc. of the organ emulsion, representing at least 5,000 minimum lethal doses. 1 hour after inoculation two of the animals were given an injection of 0.0001 cc. of the serum, and two more received an injection of 0.1 cc. The procedure was continued with each group of four animals after periods of 24, 48, and 72 hours and 4 and 5 days respectively. After 5 days the amounts of serum injected were increased to 1 and 2 cc., as it had been found that the smaller doses had no effect at this period of the disease.

All the animals which received no serum until after 72 hours showed fever at that time; in those untreated until 4 days there were fever and slight jaundice; those not treated until 5 days showed a decline in temperature and increasing jaundice; and in the group which received the injection after 6 days there were intense jaundice and subnormal temperature, and the animals were near or in collapse. The results of this series are summarized in Table III.

0.1 cc., therefore, of the anti-*icteroides* serum prevented any external manifestation of the infection if given before the onset of fever (within 72 hours after inoculation). The same dose, given to animals in the febrile stage, but without jaundice, prevented the development of jaundice; animals still having fever and showing more or less jaundice at the time of injection of this dose likewise survived, and in some jaundice rapidly disappeared in 24 hours. On the other hand, this quantity of serum failed to prevent a fatal outcome when given to animals in which defebrescence and increasing jaundice had set in. The nephritic symptoms, which had existed since 72 hours after inoculation, were rapidly increasing by this time.

0.0001 cc. of the serum prevented a fatal infection in three out of four guinea pigs when given within 24 hours. In one animal which

³ Noguchi, H., *J. Exp. Med.*, 1920, xxxi, 159.

died from the typical infection there was a prolongation of the incubation period (6 days) and the duration of illness ($5\frac{1}{2}$ days). In two of the three surviving animals fever developed after 4 days, but they recovered without showing jaundice at any time. Injection of this quantity of serum 48 hours after inoculation, or later, had no effect upon the course of the disease, all animals so treated dying within 5 to $8\frac{1}{2}$ days with typical symptoms.

Seven guinea pigs which were near or in a state of collapse 6 days after the inoculation were given 1 cc. (three animals) and 2 cc. (four animals), but with one exception all died within $\frac{1}{2}$ to $2\frac{1}{2}$ days from the time of injection of the serum.

It may therefore be concluded that the anti-*icteroides* serum here employed is able, in a dose of 0.1 cc., to protect a guinea pig from a fatal infection against at least 5,000 minimum lethal doses of the Merida strain when administered during the incubation period or at any early stage of the disease (fever and beginning of jaundice), but that it has almost no effect upon the course of the disease if given at a later period when the animals are deeply yellow and the temperature has begun to go down. At or near collapse even 1 or 2 cc. failed to prevent death in the majority of animals. On the other hand, a quantity as minute as 0.0001 cc., when given within 24 hours, is able to protect the majority of animals from a fatal infection.

If man's degree of susceptibility to *Leptospira icteroides* is comparable to that of the guinea pig, it may reasonably be assumed that the injection of the anti-*icteroides* serum at an early period of the disease will have a beneficial effect similar to that observed in the treatment of the experimental infection in guinea pigs. On the basis of body weight a man of 80 kilos would require about 200 times the amount of serum needed to save a guinea pig of 400 gm.; that is, 20 cc. ($0.1 \text{ cc.} \times 200 = 20 \text{ cc.}$). The mode of administration should be intravenous, and if necessary the injection should be repeated at short intervals (4 hours).

TABLE III.
Serotherapeutic Value of the Anti-icteroides Serum against 0.5 Cc. (5,000 Minimum Lethal Doses) of an Organ Emulsion, Merida Strain.

Length of time before serum injection. hrs.	Symptoms.	Amount of serum injected.		
		0.0001 cc.	0.1 cc.	1 cc.
1		No. 131. Incubation period 6 days. Died after 11½ days. No. 132. No infection.	No. 133. No infection. " 134. " " 137. "	2 cc.
24		" 135. Fever from 6th day on, but recovered. No. 136. Fever on 5th day, but recovered.	" 138. " " 141. " " 142. "	
48		No. 139. Died after 6½ days. No. 140. Died after 5 days.	" 145. Survived without jaundice. No. 146. Survived without jaundice.	
72	Fever; no jaundice.	No. 143. Died after 7½ days. No. 144. Fever on 5th day. Died after 7½ days.		

<p><i>days</i> 4</p>	<p>Fever; jaundice.</p>	<p>No. 147. Died after 8½ days. No. 148. Died after 6 days.</p>	<p>No. 149. Survived, but jaundice increased next day. No. 150. Survived; jaundice disappeared next day. No. 153. Died after 8½ days. No. 154. Died after 7½ days.</p>	<p>No. 159. Died after 7½ days. No. 160. Died after 8½ days. No. 161. Died after 8 days.</p>	<p>No. 155. Died after 8½ days. No. 156. Died after 6½ days. No. 157. Died after 6½ days. No. 158. Recovered.</p>
<p>5</p>	<p>Temperature down; jaundice.</p>	<p>No. 151. Died after 6 days. No. 152. Died after 5½ days.</p>			
<p>6</p>	<p>Near or in collapse.</p>				

Pfeiffer Phenomenon with the Serums of Yellow Fever Convalescents in Merida.

Pfeiffer's phenomenon with *Leptospira icteroides* had been previously observed with a limited number of serums from yellow fever convalescents in Guayaquil⁴ and was positive in about 85 per cent of the cases studied. It was desirable that this line of observation should be extended to yellow fever cases existing elsewhere, and we availed ourselves of the present opportunity with the Merida cases. Our first intention was to study as many specimens of serum from yellow fever convalescents in Merida as could be obtained, with as many strains of cultures of *icteroides* as possible. This plan could not be carried out, however, for two reasons; there were only a few convalescents accessible, and all the Guayaquil cultures brought down by us to Merida were lost within a few days after our arrival, and before our work had been started, by an accidental rise of temperature for 3 days to 56°C. of the thermostat in which the cultures had been placed for safety. Hence work on the Pfeiffer phenomenon was possible only after the isolation of *Leptospira icteroides* from Case 2.

There were two convalescents at the yellow fever hospital (Casa de Salud), Cases 2 and 3.⁵ Both patients (soldiers) were infected in the same house in Merida, Case 3 preceding Case 2 by perhaps 2 weeks. The serum for the Pfeiffer test was drawn at the end of the 5th week of the disease in Case 3 and during the 3rd week in Case 2. Both specimens were still distinctly jaundiced. Through the courtesy of Dr. A. Lara we were given specimens of serum from two other convalescents who had had a typical attack of yellow fever in August, 1919; that is, about 5 to 6 months previously. One was a young woman, whose urine still contained albumin at that time, but who showed no bile pigment either in the urine or in the serum.

The following procedure was employed. 0.5 cc. of each specimen of serum was mixed with 1.5 cc. of a pure culture of the Merida strain of the leptospira and the whole injected into the peritoneal cavity of a guinea pig. The exudate was withdrawn from the peritoneal cavity after 30 minutes for dark-field examination. Table IV is a record of the results.

⁴ Noguchi, H., *J. Exp. Med.*, 1919, xxx, 9.

⁵ Noguchi, H., *J. Exp. Med.*, 1920, xxxii, 601.

The serums from yellow fever convalescents in Merida gave uniformly a positive Pfeiffer reaction when tested with a culture of the leptospira isolated from Case 2. Although the guinea pig inoculated with the mixture of this culture and the serum from the same patient died of secondary infection too soon to permit any observation with regard to the power of the serum to protect the animal from a fatal infection, the animal which received the serum from Case 3 with this culture escaped the infection altogether. It may be supposed that this patient had been attacked by the same strain that later infected the other two soldiers (Case 1, fatal, and Case 2) living in the same

TABLE IV.

Pfeiffer Reaction with Serum from Convalescents and the Merida Strain.

Serum No.	Type of infection.	Length of time after onset of disease.	Pfeiffer reaction.	Remarks.
1 (Case 2).	Moderately severe.	<i>wks.</i> 3	Positive.	Died of intercurrent infection within 24 hrs.
2 (" 3).	Moderately severe.	5	"	No infection resulted.
3 (civilian).	Typical.	<i>mos.</i> 5	"	Died after 7 days with typical infection.
4 (").	"	6	"	Died after 7 days with typical infection.
No serum (control).			Negative.	Died after 9 days with typical infection.

house, hence such complete protection. On the other hand, Serums 3 and 4 were from two civilians who had recovered from yellow fever at least 5 months previous to the time when their blood was tested. These serums gave a positive Pfeiffer phenomenon but failed to protect the animals.

These four serums were brought back to The Rockefeller Institute to be tested with the Guayaquil strains of *Leptospira icteroides* and also with some of the strains of *Leptospira icterohemorrhagiae*. The serums from Cases 2 and 3 were accidentally lost during transportation. Moreover, during the journey the remaining two had to be

kept at a comparatively high temperature (heated steamer cabin and trains) for 15 days, and their activity must have suffered considerably. Nevertheless, tests were made on March 5; that is, 2 months from the time of collection. The amount available of Serum 3 was 0.75 cc. and that of Serum 4, 0.9 cc. Each specimen was divided into three equal parts and tested with two Guayaquil *icteroides* strains (Nos. 1 and 5) and with the American No. 1 strain of *icterohæmorrhagiæ*. The results obtained are presented in Table V.

TABLE V.

Pfeiffer Reaction with Serum from Convalescents Tested with Two Guayaquil Strains of Leptospira icteroides and One Strain of Leptospira icterohæmorrhagiæ.

Serum No.	<i>Leptospira icteroides.</i>		<i>Leptospira icterohæmorrhagiæ.</i>
	Guayaquil Strain 1.	Guayaquil Strain 5.	American Strain 1.
3	Positive.	Doubtful.	Negative.
4	Partial reaction.	Partial reaction.	"
No serum (control).	Negative.	Negative.	"

While no definite conclusions can be drawn from the results obtained with these old serums, it is possible to recognize an unmistakable specific reaction with the *icteroides* strains, particularly between Serum 3 and No. 1 of the Guayaquil *icteroides* strains. With Serum 4 there was not complete destruction of the *icteroides* organisms; but about one-half of them became paralyzed, and some were degenerated, although many active organisms were simultaneously present. There was no effect whatever upon American Strain 1 of *Leptospira icterohæmorrhagiæ*.

SUMMARY.

Identification of the leptospira isolated from a case of yellow fever in Merida was accomplished by means of an anti-*icteroides* immune serum prepared in a horse with several Guayaquil strains of *Leptospira icteroides*. The immune serum showed a protective action of high titer against the Merida strain, thus establishing its efficacy as a therapeutic agent against this strain. Polyvalent anti-*icteroides*

immune serum prepared in the horse or monovalent anti-*icteroides* immune serums prepared in the rabbit had a definite devitalizing action upon the Merida strain, while immune serums similarly prepared with strains of *icterohæmorrhagiæ* had no perceptible effect upon the Merida strain.

Serums from yellow fever convalescents in Merida gave a positive Pfeiffer reaction with the Merida strain of *Leptospira icteroides*. The reactions between the Guayaquil strains (Nos. 1 and 5) and two of these serums from convalescents varied from definitely positive to doubtful, owing probably to the diminution of active immune principles in the serums during the prolonged and unfavorable conditions of their transportation.