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IMMUNOLOGY OF THE PERUVIAN STRAINS OF LEPTOSPIRA ICTEROIDES.

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In the course of a study of the etiology of yellow fever in Peru,[1] an effort was made to solve several related problems of the disease. The first point taken up was the behavior of the serums of Peruvian convalescents in Pfeiffer's reaction towards strains of *Leptospira icteroides* isolated elsewhere; the second concerned the action, *in vitro* and *in vivo*, on the Peruvian strains of the leptospira, of a polyvalent immune serum prepared in horses with several strains of *Leptospira icteroides* of Guayaquil origin; the third related to the difference in the natural resistance to the leptospira infection of the native guinea pigs as compared with guinea pigs recently brought from New York; and the fourth and last point bore on the availability of old rabbit serum for cultivating *Leptospira icteroides*. The last two questions had arisen as a result of the rather unfavorable outcome of the work carried out in Payta, and, as will appear later, proved to be important.

Pfeiffer Reaction with Serum from Convalescents.

The method was similar to that previously employed. 1 cc. of each serum was mixed with 0.2 cc. of a rich 6 week *icteroides* culture of Guayaquil Strain 1 and the Merida strain. The guinea pigs used were those recently brought from New York. Observations were made at the end of 1 hour.[2]

[1] Noguchi, H., and Kligler, I. J., *J. Exp. Med.*, 1921, xxxiii, 239.

[2] This work was done at the Belan Hospital, Piura, where we received cordial cooperation from Dr. M. Guzman R., Dr. A. Gonzalez, and Dr. Prieto, as well as the mother superior. We also wish to thank especially Dr. Marcos L. Vega, the government sanitary officer at Piura, for the arrangement to test the convalescents in Piura.

Nine convalescents were accessible for this test. Of these, four came from the Payta (1920), four from the Piura (1919), and one from the Morropon (1920) epidemic. The serums were obtained in the five 1920 cases within a period ranging from 7 to 36 days from the time of onset of the fever and in the four 1919 cases at the end of 10 to 11 months. The results obtained are shown in Table I. The interpretation of the findings is evident. All the Peruvian cases, except two instances to be discussed below, gave a positive Pfeiffer reaction to two different strains of *Leptospira icteroides*, irrespective of whether the case occurred in Payta, Piura, or Morropon.

TABLE I.

Pfeiffer Reaction with Serum from Convalescents.

Serum No.	Locality of epidemic.	Length of time between onset of yellow fever and withdrawal of blood.	Strain of *Leptospira icteroides.*	Results.
		days		
1	Payta.	7	Guayaquil No. 1	Positive.
2	"	7	" " 1	"
3	"	36	" " 1	"
4	"	17	" " 1	"
		mos.		
5	Piura.	11	Merida.	"
6	"	10	"	"
7	"	10	"	Doubtful.
8	"	10	"	Partial reaction.
		days		
9	Morropon.	21	"	Positive.
Control.			"	Negative.

The exceptions were the serums obtained from two persons who had had yellow fever 10 months previous to the time of testing, both giving a slight or partial reaction. It may be noted, however, that two other individuals who had had an attack 10 and 11 months previously reacted positively. The reason for a partial reaction in the first two cases probably relates to a gradual diminution of the antibodies responsible for the Pfeiffer reaction.

The foregoing observations are of value in establishing the identity of the organisms that were present in individuals suffering from yellow fever in Ecuador, Mexico, and Peru. The positive Pfeiffer

reaction with the serums of yellow fever convalescents in Payta also indicates that the guinea pigs of the Payta experiments which had shown suspicious febrile reactions and hemorrhagic lesions of the lungs and kidneys without developing a typical fatal infection, had actually suffered a mild infection with *Leptospira icteroides*. Finally, it may be added that the guinea pigs which were employed in the Pfeiffer tests and reacted positively showed no symptoms and survived, while the control animal (inoculated with the Merida strain) suffered from a typical but non-fatal infection.

Virulence.

The virulence of the Morropon strains was next tested on New York guinea pigs. The material used was an emulsion of kidney

TABLE II.

Determination of the Minimum Lethal Dose of Morropon Strains.

Amount of kidney emulsion in 1 cc.	Strain 1.		Strain 3.	
	Incubation period.	Result of inoculation.	Incubation period.	Result of inoculation.
cc.	*days*		*days*	
1	4	Died in 9 days.	No test made.	No test made.
0.1	4	" " 8 "	3	Died in 8 days.
0.01	3½	" " 7 "	4	" " 9 "
0.001	5	Recovered.	3	" " 7 "
0.0001	6½	Died in 11 days.	5	" " 8 "
0.00001		No infection.	5	" " 10 "

The animals used for this experiment served at the same time as controls for the therapeutic experiments reported in Table III.

made in saline solution in a ratio of approximately 1 gm. to 10 cc. After the coarse tissue fragments had settled, the turbid supernatant portion was employed for inoculation. Ascending tenfold dilutions were prepared with 0.9 per cent saline solution, and 1 cc. was injected intraperitoneally into guinea pigs, one animal being used for each dilution. Table II gives the minimum lethal dose as determined for Morropon Strains 1 and 3. The approximate minimum lethal dose for Strain 1 lies between 0.0001 and 0.00001 cc., and that of Strain 3 beyond 0.00001 cc. The grade of virulence of the strains is, therefore, about the same as that of strains previously studied.

Therapeutic Experiments with Anti-icteroides Serum.

The reaction of the Peruvian strains towards the anti-*icteroides* immune serum prepared with the Guayaquil strains confirms the other evidence of the identity of the organisms. It has been shown in our first paper[1] that the leptospira from Peru gave a positive Pfeiffer reaction with this immune serum. In the present series of experiments the object was to determine, in guinea pigs given multiple minimum lethal doses of Morropon Strains 1 and 3, the prophylactic and curative values of a specific polyvalent anti-*icteroides* immune horse serum. The immune serum was administered in varying quantities and at different periods after inoculation, even after the appearance of symptoms, up to the time when the animals were fast approaching the terminal stage of the disease induced. The guinea pig kidney emulsions used for infecting the animals were injected intraperitoneally in uniform amounts of 0.2 cc., this dose representing at least 2,000 minimum lethal doses of Strain 1 and 20,000 minimum lethal doses of Strain 3. The immune serum was given intraperitoneally also in doses of 0.00001, 0.0001, 0.001, 0.01, and 0.1 cc., contained in saline solution to a total volume of 1 cc., and finally 1 cc. of undiluted serum. In order to obviate the factor introduced by an occasional unusually refractory animal, more than two of the recently brought guinea pigs were used in testing the effect of each dose of serum. Table III, which summarizes the results, brings out the following points. (*a*) The anti-*icteroides* serum is capable of checking the development of the infection, provided a sufficiently large quantity is given during the incubation period. The four deaths which took place among animals treated during this period occurred among those which did not receive serum until 72 hours after inoculation; *i.e.*, at the end of the incubation period. (*b*) A quantity of serum larger than 0.01 cc., given during the febrile stage (96 hours after inoculation) usually prevented the infection from reaching the icteric stage. This was true particularly with the animals infected with Strain 1, which had scarcely a tenth of the virulence of Strain 3. (*c*) The effect of the serum became uncertain when it was given during the icteric stage (5 to 6 days after inoculation). With 0.01 cc. no animal was saved, while 0.1 cc. saved three out of

four infected with Strain 3, but none with Strain 1. 1 cc. of undiluted serum saved one out of three with Strain 1 and three out of four with Strain 3. That the proportion of recoveries among the animals treated during the icteric stage does not correspond with the virulence of the strains can be explained only on the basis of the natural resistance of individual guinea pigs; at all events, death occurred in both groups, even when 1 cc. of serum was given.

TABLE III.

Protective and Curative Effect of Anti-icteroides Serum in Guinea Pigs Experimentally Infected with Leptospira icteroides.

Strain.	Amount of serum.	Animals treated before beginning of fever.			Animals treated during febrile stage.			Animals treated after jaundice appeared.		
		Total.	Remained well.	Died.	Total.	Recovered without jaundice.	Died.	Total.	Recovered.	Died.
	cc.									
0.2 cc. of Strain 1 culture, representing 2,000 minimum lethal doses.	0.0001	2	1	1						
	0.001	5	5	0	3	0	3			
	0.01	4	4	0	5	5	0	2	0	2
	0.1	4	4	0	3	3	0	2	0	2
	1				4	4	0	3	1	2
0.2 cc. of Strain 3 culture, representing 20,000 minimum lethal doses.	0.0001	2	2	0						
	0.001	6	5	1	2	0	2			
	0.01	6	4	2	5	3	2	2	0	2
	0.1	3	3	0	3	3	0	4	3	1
	1				2	2	0	4	3	1

The controls without serum treatment are represented by the animals of the experiment recorded in Table II.

Resistance of Native and Imported Guinea Pigs to Leptospira icteroides.

Certain tests made with guinea pigs obtained in Payta suggested that the native Peruvian guinea pigs are more refractory to the leptospira than guinea pigs brought from New York. To determine this point four groups of guinea pigs were inoculated, on May 16, 1920, with a uniform amount of kidney emulsion from a guinea pig which had shown a typical infection with Morropon Strain 4. The

animals inoculated comprised: (1) fifteen native guinea pigs which had once been inoculated with culture materials in Payta, without any definite infection having been induced; (2) six native guinea pigs which had not been used; (3) eight American guinea pigs which had formerly been inoculated with culture materials in Payta, without infection having been induced; and (4) four newly imported American guinea pigs. Table IV summarizes the result. It will be noted that the virulence and dose of the strain used were such that even the young American guinea pigs, used as a standard of susceptibility, did not all develop a fatal infection. This effect was rather an advantage, since, had multiple fatal doses been given, the difference in susceptibility might not have come out so clearly.

TABLE IV.

Relative Resistance of Native and American Guinea Pigs to Leptospira icteroides.

Guinea pigs.	Total No. injected.	Escaped infection (no jaundice).	Animals showing definite infection.*	
			Recovered.	Died.
Native (once used)	15	9	1	5
" (normal)	6	6		
American (old lot; once used)	8	3	3	2
" (new lot; normal)	4	0	2	2

* This includes guinea pigs which showed typical lesions when killed.

The striking feature of the foregoing experiment is the complete resistance of all six normal native guinea pigs in contrast to the four normal American animals, of which two, at least, died of a typical infection. Among the animals inoculated on a former occasion, both the native and the American groups contained susceptible as well as refractory ones, the difference being that a larger proportion of the native (60 per cent) than of the American (37.5 per cent) proved resistant.

The relatively large proportion of resistant animals among the native guinea pigs, as well as among the American guinea pigs which had withstood the unfavorable climate and other hardships, is a point to be taken into account in future work.

Deterioration of Rabbit Serum through Age.

That an alteration takes place in rabbit serum when it is subjected to long transportation in a hot climate is evident from the fact that no growth of *Leptospira icteroides* could be obtained with such serum. On the other hand, old rabbit serum which has been kept in the refrigerator up to 6 weeks has been found still suitable for making subcultures of *icteroides*. Whether the same would be true after 2 or 3 months standing at 4°C. has not, however, been determined.

The rabbit serum which was used in the cultivation experiments in Payta[1] was 3 months old and had been exposed to the ordinary tropical temperature of the region, which is sometimes 40°C. during the day, and it contained considerable precipitate. It was thought probable that the deterioration of the serum through age and climatic conditions might have been a factor in the unsuccessful outcome of the Payta experiments. Moreover, another lot of serum, which had been collected in New York in April and hermetically sealed, was brought down to Piura under ordinary temperature conditions. Some tubes contained precipitate, while others had remained clear. It was used when 50 days old for making subcultures of four Morropon strains of the leptospira, but when the culture tubes were examined 10 days later no growth could be found. Fearing a possible loss of all strains, we made two more sets of subcultures of the same strains, one set with the old and another with fresh rabbit serum. With the media containing the fresh rabbit serum a rich growth took place with three of the four strains, but there was no growth in the tubes prepared with the old serum.

Other factors, however, made it difficult to determine how important it is in general to employ fresh serum in culture work with *icteroides*. The Peruvian strains were unusual in that they early degenerated in culture. The initial cultures from human cases never became very rich, and they disappeared within about 3 weeks. The first generation cultures from the infected guinea pigs grew fairly well within a week, but at the end of 2 weeks examination showed only a few active organisms and many degenerating. Of the cultures of Strain 3, none was alive, and injection into guinea pigs failed to recover it. The remaining strains (Nos. 1, 2, and 4) were successfully

subcultured by using fresh rabbit serum, as noted above. It is certain that all the strains would have been lost if we had relied entirely on the old serum. The present study does not indicate whether the importance of using fresh serum applies only to the primary human and recently isolated generations or even to remote subcultures.

SUMMARY.

Serum from yellow fever convalescents from Payta, Piura, and Morropon gave a positive Pfeiffer reaction with the strains of *Leptospira icteroides* isolated in Guayaquil and Merida. The serum also protected the guinea pigs from these strains in the majority of instances. The Pfeiffer reaction was complete with all recent convalescents (7 to 36 days) but slight or partial in some instances with serum derived from individuals who had had the attack of yellow fever 10 months previously.

The virulence of the Morropon strains was found to be approximately the same as that of the Guayaquil or Merida strains. With one strain the minimum lethal dose for the guinea pig was less than 0.00001 cc. of a kidney emulsion from an infected guinea pig.

Suitable quantities of the anti-*icteroides* serum administered to guinea pigs inoculated with 2,000 to 20,000 minimum lethal doses of infective material prevented the development of the infection, or a fatal outcome, according as the serum was given during the incubation period or after fever had appeared. The earlier the administration of the serum the smaller was the quantity needed; during the incubation period 0.0001 to 0.001 cc. was sufficient, during the febrile period 0.01 to 0.1 cc. was required to check the progress of the disease, and even at the time when jaundice had already appeared, the injection of 0.1 to 1 cc. saved three out of four animals inoculated with Strain 3 and one out of three inoculated with Strain 1.

The native guinea pigs secured in Payta proved to be unusually refractory to infection with *Leptospira icteroides* as compared with normal guinea pigs recently imported from New York.

Fresh rabbit serum is recommended for culture work with *Leptospira icteroides.*