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A COMPARATIVE STUDY OF EXPERIMENTAL PROPHYLACTIC INOCULATION AGAINST LEPTOSPIRA ICTEROHÆMORRHAGIÆ.

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Ido, Hoki, Ito, and Wani[1] first demonstrated that when guinea pigs are treated with three successive injections of killed cultures of *Leptospira icterohæmorrhagiæ* at 5 day intervals they become refractory to a subsequent infection, undertaken 10 days later, with a virulent culture or with liver emulsions of infected guinea pigs. After about 17 to 19 days the serum of these animals contained a small amount of immune bodies when tested by the method of Pfeiffer. From this experiment the conclusion was drawn that the degree of immunity induced in these animals is comparable with that which develops in the serum of patients on the 8th day of disease, in the stage when the infective organisms have already disappeared from the peripheral blood, as shown by negative results of animal inoculations with the blood. The immunity is incomplete, but sufficiently strong to protect the animals from the infection. These writers found also that the serum of six persons inoculated with three successive doses of highly concentrated vaccine (carbolized emulsion of the pure culture) for the purpose of prophylaxis contained a demonstrable amount of immune bodies when tested by Pfeiffer's method 10 days after the last inoculation, but when given to unvaccinated guinea pigs the serum failed to protect the animals against a simultaneous infection. There was considerable difference, however, between the course of the disease in the animals receiving the serum from vaccinated persons and those receiving normal human serum, the former outliving the controls by as many as 10 days, the number of spirochetes being fewer, and the icterohemorrhagic lesions less marked.

Ito and Matsuzaki[2] recommended for the purpose of protective vaccination the use of the blood gelatin culture of *Leptospira icterohæmorrhagiæ*. The culture was killed by heating it to 60°C. for 30 minutes on 3 successive days. The killed culture was toxic when given intravenously, and one of the samples killed guinea pigs, in a dose of 0.5 cc. or more per 100 gm. of body weight, within a few minutes. A single subcutaneous injection of 0.05 cc. of another preparation of the killed cul-

[1] Ido, Y., Hoki, R., Ito, H., and Wani, H., The prophylaxis of Weil's disease (spirochætosis icterohæmorrhagica), *J. Exp. Med.*, 1916, xxiv, 471.

[2] Ito, T., and Matsuzaki, H., Prophylactic vaccination, serotherapy, and chemotherapy in Weil's disease (in Japanese), *J. Chiba Med. College*, 1916, Nos. 83 and 84.

ture, the minimum lethal dose of which was 1.5 cc. per 100 gm. of body weight, was found to be sufficient to confer upon the animal a complete immunity against a subsequent infection after 2 weeks, although there was no protection at the end of the 1st week. Ito and Matsuzaki also prepared a pulverized vaccine from a desiccated blood gelatin culture of the *icterohæmorrhagiæ* and found that it not only retained its immunizing quality but possessed the advantage over the liquid vaccine of being nearly ten times more concentrated. For protective vaccination they used a saline emulsion of the dried culture. They calculated the quantity necessary for a man of 50 kilos body weight to be 25 to 30 cc. of the liquid and 2.5 to 3 gm. of the dried material. The serum from the vaccinated animals contained the specific immune bodies demonstrable by Pfeiffer's procedure and also *in vitro* (Sabritschewsky's phenomenon).

From the experiments of Ido, Hoki, and others on the one hand, and those of Ito and Matsuzaki on the other, it is evident that the guinea pig, which is perhaps the animal most susceptible to infection by *Leptospira icterohæmorrhagiæ*, can be rendered actively immune by a single injection or by multiple injections of devitalized cultures. The question arises as to whether the immunity thus induced with one strain will protect the animal equally well against the other strains. The present work was undertaken to determine the relation of one strain to another by means of cross-immunity reactions among the strains isolated in Japan, America, and Europe.

Method.

For the purpose of protective inoculation guinea pigs weighing about 400 gm. were selected. The emulsions of *Leptospira icterohæmorrhagiæ* were prepared from pure cultures of different strains grown on the rabbit serum Ringer medium (semisolid below and liquid above[3]) at 26°C. for from 3 to 6 weeks. The liquid portion of the culture, showing 20 to 30 organisms per field ($\frac{1}{12}$ oil immersion and Leitz ocular 3), was gathered from a number of tubes and, after immobilization of the organisms by means of 0.4 per cent phenol (determined by the dark-field microscope), was centrifuged for 2 hours at the rate of 4,000 revolutions per minute; the deposit was carefully separated from the supernatant fluid and resuspended in one-fifth

[3] Noguchi, H., Further study on the cultural conditions of *Leptospira (Spirochæta) icterohæmorrhagiæ*, *J. Exp. Med.*, 1918, xxvii, 593.

of the original volume. The emulsion thus concentrated contained a great number of the organisms and was preserved under a layer of toluene for 1 week in the refrigerator (6°C.) before use. The emulsion was then tested by culture in order to be certain that there were no live organisms left.

Emulsions were prepared from two American strains isolated from wild rats caught in the vicinity of New York, one European, and one Japanese strain,[4] and the inoculations were made subcutaneously. In the first group of animals 0.5 cc., in the second 0.05 cc., and in the third 0.005 cc. of the emulsion was given three times at intervals of 5 days. Except in a few instances the animals showed no rise in temperature after the inoculation. As a whole they stood the inoculations well, though a number were lost through secondary infection. Some lost weight during the vaccination period.

Tests for active immunity against the homologous and heterologous strains were made at the end of 2, 4, and 8 weeks after the last inoculation. The quantities used for the injection intraperitoneally into the vaccinated and control (unvaccinated) animals were those containing at least several minimum lethal doses of each strain. For example, in the case of American Strain 1, 0.0005 cc. was used, American Strain 2, 0.005 cc., American Strain 3, 0.05 cc., Japanese, 0.00005 cc., and European, 0.005 cc. of 3 week cultures on the rabbit serum Ringer medium at 26°C. Two control animals accompanied each series of experiments, and they always died of the typical infection within 5 to 7 days.

Notwithstanding the care which they received, some of the vaccinated guinea pigs died of secondary infection, or in a few instances of marasmus, before the experiments were completed.

RESULTS.

The results of the tests are briefly summarized in Tables I to IV.

Table I shows that the guinea pigs receiving three successive injections of 0.05 and 0.5 cc. of the concentrated vaccine of American Strain 1 were, with two exceptions, protected against a subsequent

[4] Noguchi, H., *Spirochæta icterohæmorrhagiæ* in American wild rats and its relation to the Japanese and European strains, *J. Exp. Med.*, 1917, xxv, 755.

TABLE I.

Vaccine Made from American Strain 1.

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
American Strain 1	Survived.	Survived.	Died in 7 days.	Died in 6 days.	Survived.	—	Survived.	Died in 6 days.	—	Survived.	Survived.	Died in 7 days.
" " 2	"	Died in 8 days.	Died in 9 days.	Died in 6 days.	"	Survived.	Died in 7 days.	Died in 6 days.	Survived.	"	Died in 8 days.	Died in 6 days.
" " 3	"	Survived.	Died in 8 days.	Died in 5 days.	—*	"	Survived.	Died in 6 days.	"	—	Survived.	Died in 7 days.
Japanese strain.	"	"	Died in 8 days.	Died in 6 days.	Survived.	"	Died in 11 days.	Died in 5 days.	"	Survived.	Died in 7 days.	Died in 5 days.
European "	"	Died in 8 days.	Died in 8 days.	Died in 7 days.	"	"	Died in 8 days.	Died in 6 days.	"	"	Died in 8 days.	Died in 6 days.

*— indicates that the animal succumbed to secondary infection.

TABLE II.

Vaccine Made from American Strain 2.

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0 005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
American Strain 2	Survived.	Survived.	Died in 7 days.	Died in 6 days.	Survived.	Survived.	Died in 9 days.	Died in 7 days.	—	Survived.	Died in 9 days.	Died in 6 days.
" " 1	"	"	Died in 8 days.	Died in 6 days.	"	"	Died in 8 days.	Died in 6 days.	Survived.	Died in 13 days.	—	Died in 6 days.
" " 3	"	"	Died in 9 days.	Died in 7 days.	"	"	Died in 7 days.	Died in 7 days.	"	—	Died in 7 days.	Died in 7 days.
Japanese strain.	"	Died in 10 days.	Died in 7 days.	Died in 5 days.	"	—	Died in 8 days.	Died in 5 days.	"	Died in 10 days.	Died in 6 days.	Died in 5 days.
European "	"	Survived.	Died in 8 days.	Died in 7 days.	"	Survived.	Died in 8 days.	Died in 6 days.	"	Survived.	Died in 8 days.	Died in 6 days.

TABLE III.

Vaccine Made from Japanese Strain.

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
Japanese strain.	Survived.	Survived.	Died in 11 days.	Died in 5 days.	Survived.	Survived.	Survived.	Died in 5 days.	Survived.	—	Survived.	Died in 5 days.
American Strain 1	"	"	—	Died in 6 days.	—	"	Died in 9 days.	Died in 6 days.	"	Survived.	Died in 8 days.	Died in 6 days.
" " 2	"	—	Died in 9 days.	Died in 7 days.	Survived.	"	Died in 10 days.	Died in 7 days.	—	Died in 9 days.	Died in 11 days.	Died in 7 days.
" " 3	"	Survived.	Died in 7 days.	Died in 6 days.	"	"	Died in 12 days.	Died in 6 days.	Survived.	Survived.	Died in 13 days.	Died in 6 days.
European strain.	"	Died in 8 days.	Died in 8 days.	Died in 6 days.	"	"	Died in 8 days.	Died in 6 days.	"	Died in 12 days.	Died in 8 days.	Died in 6 days.

TABLE IV.

Vaccine Made from European Strain.

Strain.	Period after last inoculation.											
	2 wks.				4 wks.				8 wks.			
	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.	0.5 cc.	0.05 cc.	0.005 cc.	Control.
European strain.	Survived.	Survived.	Died in 8 days.	Died in 5 days.	Survived.	Survived.	Survived.	Died in 6 days.	Survived.	Survived.	Died in 12 days.	Died in 6 days.
American Strain 1	"	Died in 12 days.	Died in 8 days.	Died in 6 days.	—	"	Died in 8 days.	Died in 6 days.	"	Died in 9 days.	Died in 8 days.	Died in 6 days.
" " 2	"	Survived.	Died in 9 days.	Died in 6 days.	Survived.	"	Died in 13 days.	Died in 6 days.	—	Survived.	Died in 9 days.	Died in 6 days.
" " 3	"	"	Died in 13 days.	Died in 6 days.	"	"	Died in 8 days.	Died in 6 days.	Survived.	"	—	Died in 7 days.
Japanese strain.	"	Died in 8 days.	Died in 6 days.	Died in 5 days.	"	—	Died in 6 days.	Died in 5 days.	"	Died in 8 days.	Died in 6 days.	Died in 5 days.

infection undertaken 2, 4, and 8 weeks after the last inoculation. One exception occurred in the case of American Strain 2 and one in the case of the European strain, both animals succumbing to the infection 1 to 2 days later than their respective controls. On the other hand, three injections of 0.005 cc. of the same vaccine protected the animals against the same strain as well as against American Strain 3, but failed to produce complete immunity against the other three heterologous strains.

Table II gives the results obtained with the guinea pigs vaccinated with American Strain 2. The protection was complete in the group receiving 0.05 and 0.5 cc., except when tested against the Japanese strain and American Strain 1, in which instances death was delayed but not prevented. There was no resistance against any strain when the dose of the vaccine was 0.005 cc.

In the series reported in Table III, the highest degree of immunity is seen to have been developed against the Japanese strain, which was the one used as the vaccine in this series. It was the only strain which failed to kill guinea pigs that received injections of the smallest amount of the vaccine (0.005 cc.). Protection was weakest against American Strain 2 and the European strain. Three injections of 0.5 cc. in succession, however, conferred complete immunity against all the strains tested.

A similar relation exists between the homologous and heterologous strains of the next series (Table IV), the protection being most marked against the same organism. All the guinea pigs vaccinated with 0.5 cc. of any one strain resisted infection with that and all other strains used in the present study.

While these results do not furnish basis enough to permit division of the various strains into groups or definite types, yet the general indication is that there is a closer affinity among some strains than others. For example, American Strains 1 and 3 seem to be closely allied, American Strain 2 being nearer to the European strain. The Japanese strain stands between the two groups and much nearer that formed by American Strains 1 and 3.

With respect to the duration of the immunity thus established, it was found that at the end of 8 weeks there was not an appreciable diminution of the immunity, although of the animals receiving smaller amounts of the vaccine a slightly larger number survived when tested at the end of 4 than at the end of 2 or 8 weeks.

SUMMARY AND CONCLUSIONS.

Guinea pigs were inoculated with suspensions of *Leptospira icterohæmorrhagiæ* obtained from pure cultures of several different strains, in order to determine whether or not an active immunity against a subsequent infection with virulent organisms would develop in the vaccinated animals. The experiments were so arranged as to make possible a determination of the existence of immunity against homologous strains as well as against the strains not employed as vaccine, and a brief quantitive estimation of the degree and duration of the immunity in relation to the quantities of the vaccines inoculated. Following the general rule of prophylactic inoculations with various pathogenic organisms, the inoculations were repeated subcutaneously on three consecutive occasions at intervals of 5 days. With respect to the amounts of vaccine, the experiments were divided into three groups for each vaccine, one group receiving three doses of 0.5 cc., the second three of 0.05 cc., and the third three of 0.005 cc. Four different strains were employed as vaccines, American Strain 1, American Strain 2, and one each of the Japanese and the European strains.

The determination of the development, degree, and duration of the immunity was made by inoculating intraperitoneally several minimum lethal doses of each of the five following strains: American Strains 1, 2, and 3, the Japanese, and the European strains. The virulence of the different strains varied considerably, the strongest being the Japanese strain, which killed the guinea pig in a dose of 0.00001 cc., and the weakest American Strain 3, the minimum lethal dose of which was as large as 0.01 cc.

The vaccinated guinea pigs were tested for immunity at the end of 2, 4, and 8 weeks after the last inoculation.

The results obtained show that three successive inoculations of 0.5 cc. of the emulsions of killed cultures of *Leptospira icterohæmorrhagiæ* into guinea pigs rendered them completely resistant to a subsequent infection with the virulent cultures of both homologous and heterologous strains. With 0.05 cc. the protection was not so general, the animals succumbing to an experimental infection with some heterologous strains while resisting the homologous and other heterologous strains. The animals which were vaccinated with 0.005 cc. survived

the infection experiments with the homologous strains in the case of American Strain 1 and the Japanese strain, but they were not protected against any other strains. The vaccines of other strains were unable to immunize the guinea pigs so highly even against their homologous strains, when the amount of each inoculation was only 0.005 cc., but 0.05 cc. conferred complete protection against the same strains. It may be concluded, therefore, that when a sufficient quantity of killed cultures of *Leptospira icterohæmorrhagiæ* is given, the guinea pigs will become immune to all strains of the same organism, but that smaller quantities may protect them against homologous but not against heterologous strains. A close analysis reveals the existence of group or type affinities among different strains which can be brought out by immunizing the animals with smaller quantities of killed cultures. In the present series of experiments American Strains 1 and 3 form one group, American Strain 2 and the European strain another, and the Japanese strain a third, which is also closely allied to the first group.

In order to insure universal immunity it is wise to employ as many group or type cultures as possible in the preparation of vaccines, a polyvalent vaccine being recommended. It is not improbable that the strain recently encountered in Lorient, France,[5-8] is an unusually deviated type of *Leptospira icterohæmorrhagiæ*, and that if successfully cultivated and used as vaccine in sufficient amount it might protect the animals against other strains of the same organism.

The active immunity induced in the vaccinated guinea pigs was found to persist for at least 8 weeks after the last inoculation. It will no doubt last for a much longer period.

[5] Manine, Cristau, and Plazy, *Compt. rend. Soc. biol.*, 1917, lxxx, 531.

[6] Manine and Cristau, *Bull. et mém. Soc. méd. hôp. Paris*, 1917, xli, series 3, 977, 1045.

[7] Pettit, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 774.

[8] Pettit, A., *Compt. rend. Soc. biol.*, 1918, lxxxi, 48.