

## STUDIES ON TYPHUS FEVER

### X. FURTHER EXPERIMENTS ON ACTIVE IMMUNIZATION AGAINST TYPHUS FEVER WITH KILLED RICKETTSIA\*

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In a number of preceding communications, the writers have published the results of active immunization of guinea pigs with formalin-killed *Rickettsiae* obtained from the tunica lesions of guinea pigs infected with the Mexican virus. Such experiments showed that it was possible to obtain a considerable degree of active immunity against infection with the Mexican virus. Against the European virus this method gave only a partial immunization, amounting often only to a distinct diminution of the severity of the disease.

Since the publication of our earlier results, the methods of vaccine production have been considerably improved by the use of rats in which resistance to typhus had been depressed either by benzol injection or by exposure to radiation with short wave length X-rays. The X-ray method has since been found to be far the more regularly successful one. Intraperitoneal inoculation of animals so treated with moderate doses of Mexican virus yields almost invariably massive amounts of peritoneal *Rickettsiae* (1). This method is now being extensively investigated by Varela and others in Mexico, and its effectiveness in man will, we believe, eventually be determined by these workers. Meanwhile, we have been induced to carry out a new series of immunization experiments on guinea pigs with the use of the X-rayed rat vaccines, since in these preparations there is a much higher concentration of *Rickettsiae* than in any of the others hitherto employed by us.

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For active immunization in typhus fever we have at the present time a limited choice of possibilities. In an earlier paper with Batchelder one of us has shown that effective active immunization can be carried out with mixtures of convalescent serum and living virus—a method too perilous to be justified for use in man if there is any possibility of accomplishing the same purpose with dead materials. For immunization against the European disease, the carbolized louse vaccines of Weigl (2) seem to have been effective, but it is obvious that the technical difficulties involved in the use of insects considerably complicate practical application. The same objection applies to the recent method described by Dyer and his collaborators (3) in which a vaccine against the endemic typhus was produced from fleas infected with the virus. That immunization is possible by the method originally described by us has been confirmed by Kemp (4), who, however, found that the immunity was not lasting and that the vaccine retained its potency for a short time only. Dyer and his collaborators cite this paper in criticism of our method without, however, considering that Kemp's experiments were carried out with the original, unimproved technique described by us, in which the vaccination material contained relatively few *Rickettsiae* as compared with our later methods. Moreover, the results reported by Dyer in his experiments were in no respect better than those obtained in some of our series, a fact recognized by Dyer, who believes that a better vaccine than he has so far obtained could be prepared eventually by the flea method.

Since the experiments of Weigl, as well as our own, seem to have demonstrated conclusively that immunity in these diseases can be obtained with dead virus, the ideal method would be one in which sufficient amounts of the *Rickettsiae* could be obtained either by direct cultivation or by tissue culture. Direct cultivation has not so far been possible and tissue culture, though easily accomplished, has not as yet yielded adequate amounts of the organisms. We believe that the easiest procedure so far available by which a sufficiently large amount of vaccine can be produced with the Mexican material is the X-rayed rat method described by us. Under conditions of reasonable success, about 15 cc. of a suspension of washed *Rickettsiae*—almost comparable to typhoid vaccine in the number of organisms—can be obtained from a single rat.

The purpose of the present communication is to add to our previous reports the results of active immunization in guinea pigs with this X-rayed rat material killed with 0.2 per cent formalin. We are not at all sure that formalin is better than phenol for preservation, but we have not had time so far to carry out comparative experiments.

#### EXPERIMENTAL

*Preparation of Animals.*—Guinea pigs were vaccinated with formalinized rat *Rickettsia* made by the X-ray method containing a relatively small amount of detritus but very large amounts of *Rickettsiae*. The vaccine was not used immediately after being made, but had been kept from 1 to 3 weeks in the ice box.

Fifteen guinea pigs received the vaccine intraperitoneally, and five guinea pigs received it subcutaneously, as follows:

|             |     |
|-------------|-----|
| May 18..... | cc. |
| “ 23.....   | 0.5 |
| “ 28.....   | 1.0 |
| June 3..... | 1.0 |

No protection experiments were done until July 9, it being desirable for practical purposes to determine whether any acquired immunity would last for at least a minimum of 5 weeks and longer.

*Experiment I. July 9, 1932. Inoculation with Mexican Typhus Virus.*—The infectious material for this experiment was the tunica scrapings of two guinea pigs, Nos. 1 and 2, killed on the 6th day after intraperitoneal inoculation with tunica and blood from an animal infected with Mexican virus. Three vaccinated animals and two controls received, each, 2 cc. of a suspension of the four tunicas, the dose representing about one-third of a tunica for each guinea pig. Examination of the tunicas after the experimental inoculations had been made revealed that in one of them there were enormous numbers of extracellular *Rickettsiae*, far more than are usually seen in guinea pigs at this stage. These animals, therefore, received tremendous doses. The vaccinated guinea pigs weighed from 80 to 125 gm. more than the two controls, although the largest available control animals were chosen.

The results of this experiment are unsatisfactory, perhaps because of the enormous dosage employed. Nevertheless, there was distinct evidence of a higher resistance in the vaccinated than in the control animals. The controls on the 4th day reached temperatures of 104.2° and 106°, respectively, and typical swelling appeared at that time. One of these control animals, No. 4, was killed on the 6th day, in order to keep the strain going. Its temperature had not gone below 104° since the 4th day and was at that time 104.5°. The other control ran a temperature fluctuating between 105° and 106° from the 4th to the 8th day, with swelling that did not decrease until the 8th and 9th days.

Of the vaccinated pigs, one animal seemed to be completely protected, no swelling occurring at any time, and the temperature remaining at or below 103°, except on the 6th day, when it touched 104° in the afternoon. The subcutaneously vaccinated guinea pig developed no swelling until the 6th day, when the temperature touched 105°. The temperature remained at 104° only 2 days, then promptly dropped to normal. The third vaccinated animal, though running a typical temperature, did not develop swelling until the 6th day, and ran a course much less violent than the control that had been allowed to survive.

There was thus distinctly less severity of the disease in two of the vaccinated guinea pigs, and apparently complete protection in one of them. This protected animal, however, is the only one of the series to which we would attach much importance, since the milder nature of the disease in the others might well have been due to the greater weight of the vaccinated animals. We omit charts, as unnecessary for the description of these experiments.

*Experiment II. July 9, 1932. Vaccinated Animals Inoculated with Mexican Typhus Blood.*—Since we realized at the time that we were doing Experiment I that we were giving an enormous amount of virus, and since we had bled and defibrinated the blood of the two guinea pigs that had furnished the tunicas, we carried out, on the same day, another experiment in which we inoculated intraperitoneally two controls, two intraperitoneally vaccinated animals and one which had been subcutaneously vaccinated with 1 cc. of the mixed, defibrinated blood specimens of the Mexican typhus Guinea Pigs 1 and 2, described in Experiment I. Again there was, unfortunately, a considerable discrepancy in weight between vaccinated animals and controls, the vaccinated guinea pigs all weighing between 680 and 750 gm. each, while the controls weighed 550 and 650 gm., respectively.

In this experiment, as was to be expected, the controls did not develop typhus until much later than they would have had they been inoculated with tunica material. The temperatures did not rise above 104° in the controls until the 8th day and the 11th day, respectively. Swelling did not appear until the 11th and the 12th days, respectively, but was then typical, and *Rickettsiae* were found on the 13th day in one of these animals, killed for transfer. Both of them, in other words, ran late but typical Mexican typhus fever. The three vaccinated animals developed no swelling whatever, though they were observed for 15 days. One of them—and, perhaps significantly, the subcutaneously vaccinated one—never reached a temperature higher than 103°. One of the intraperitoneally vaccinated animals touched 104° for 1 day, the 14th; the other reached this temperature on the 8th and 12th days, respectively, promptly returning to normal in each case. In none of the vaccinated guinea pigs was there any evidence of typhus fever except the slight, temporary temperature rises mentioned above. In none of them did scrotal swelling develop.

We feel safe in assuming that in this experiment, with a moderate dose of infectious material, we obtained practically complete immunity in all three of the vaccinated animals.

*Experiment III. July 18, 1932. Vaccinated Animals Inoculated with Tunica Virus, Eastern United States Variety (Maxcy).*—This experiment was carried out with the Wilmington strain of typhus, that is, the variety discovered by Maxcy to exist in the eastern United States. This disease may be regarded as identical with the Mexican type.

The source of material for infection was tunica material, showing *Rickettsiae*, from a guinea pig inoculated on July 8 with frozen spleen preserved by Dr. Pinkerton at a temperature below zero for about a week. This source animal showed a typical reaction on the 8th day. When killed for removal of the tunica on the 10th day, there was swelling and a temperature of 105°.

Two controls and two treated animals—one vaccinated subcutaneously, the other intraperitoneally—were intraperitoneally inoculated with about one-quarter of a tunica, the material containing relatively few *Rickettsiae*. Apparently the dose was larger than we suspected from the appearance of the tunica, and a tempestuous disease occurred in the two normal controls. These animals developed temperatures of 104° and 105°, respectively, on the 3rd day. This was followed by the customary remission on the 4th day and a return to temperatures above 104° on the 5th day. One of the controls developed typical swelling on the 6th day, the other not until the 8th day, the course of the disease from then on being in every way characteristic. The two vaccinated animals showed no signs of typhus fever. One of them was observed for 2 weeks, during which the temperature rarely went above 103°, never above 103.5°, and there was no swelling at any time. The other touched 104° on the 6th day, but promptly came down and stayed down until the 11th day, a time at which the disease had passed through its entire cycle in the controls. A rise of temperature to 104° on the 12th day in one of these animals was shown to be due to intercurrent pneumonia.

We believe that this experiment indicates complete protection by the vaccine. But again in this experiment we were forced by circumstances to choose controls that were from 100 to 150 gm. lighter than the vaccinated guinea pigs, which had grown heavier during the interval between vaccination and protection tests.

*Experiment IV. July 29, 1932. Inoculation with Mexican Tunica of Animals of Approximately Equal Weight.*—Although past experience has not indicated that, after guinea pigs have reached a weight of 400 or 500 gm., there is any noticeable difference between the large and the smaller ones in susceptibility to typhus infection, nevertheless, we were still a little troubled by the fact that in all the experiments so far recorded we had been forced by circumstances to use controls

that were lighter than the vaccinated animals. We therefore procured a number of control animals that corresponded in weight more closely to the vaccinated guinea pigs.

The source of material for the experiment done on July 29 was tunica material from a Mexican typhus guinea pig killed on the 6th day, with typical swelling and a moderate number of *Rickettsiae* in the tunica. Each animal in the experiment was injected intraperitoneally with what amounted to approximately a fifth of a tunica, which should represent several hundred times the minimal infectious dose, although the irregularity of the distribution and amounts of *Rickettsiae* in individual tunicas is such that it would be absurd to attempt definite statements in this regard. In the past, we have obtained infection with 1/1000th of a tunica of the ordinary passage animal.

The animals were injected as follows:

|                |         |     |     |
|----------------|---------|-----|-----|
| Control 1..... | weight, | gm. | 750 |
| “ 2.....       | “       | “   | 680 |
| “ 3.....       | “       | “   | 720 |
| “ 4.....       | “       | “   | 520 |

(Control 4 was added for the sake of passing the strain, and giving us some idea as to the difference in susceptibility dependent upon differences of weight at this range.)

|  |         |     |     |
|--|---------|-----|-----|
| Intraperitoneally vaccinated guinea pig..... | weight, | gm. | 910 |
| “ “ “ “.....                                 | “       | “   | 700 |
| Subcutaneously “ “ “.....                    | “       | “   | 800 |

None of the vaccinated guinea pigs developed typical typhus fever. Two of them—one, perhaps significantly, the subcutaneously vaccinated one—never exceeded a temperature of 103.5°, and were below this for all but 1 day. None of the vaccinated animals developed swelling. The third animal, and—fortunately for conclusions—the heaviest, had an isolated rise of temperature to 105° on the 6th day, and touched 104° on the 8th, but at no time had swelling. This may perhaps indicate a very mild typhus reaction.

The controls all ran typical Mexican fever of a severe type. There was no great difference in the severity of the disease sustained by the lighter control and that occurring in the heavier guinea pigs. In all four controls the temperature was either 105° or almost that on the 4th day, and evidence of swelling was present in all of them on that day, the large as well as the small.

It seems a fair conclusion that the vaccine completely protected two of our animals against the virus given in this experiment and almost completely protected in the third. Also, the very slight difference in the severity of the disease in the light and the heavy controls lends more weight to our previously reported experiments, where this point was the only obvious source of error.

The foregoing experiments seem to us to furnish adequate proof that our formalinized *Rickettsia* vaccines are capable of producing an active immunity against the Mexican typhus virus as well as against the endemic typhus of Maxcy, provided reasonable doses of the infectious material are used for the protection tests. But even when, as in Experiment I, an overwhelming dose of *Rickettsiae* is administered, there is a prolongation of incubation time, a modification of the disease in the direction of mildness and, occasionally (one animal of three), complete protection.

Although there is an unquestionably close relationship between the infectious agents of the New World typhus and those causing the European disease—as indicated by cross-immunity and overlapping agglutination reactions—there are, nevertheless, considerable differences between the two diseases which prohibit the assumption that a method of vaccination effective against one disease will be equally effective against the other. Recent investigations by Varela and his collaborators (5) appear to indicate that the vaccine prepared by our method is protective against the Tunisian virus. But this virus, as sent to us by Dr. Nigg, has in our hands shown the characteristics of the Mexican-American type,—rapid development of temperature and frequent scrotal swelling. Whether it has become modified since importation, or whether it is intrinsically different from the typical European virus, we are unable to state at present. Meanwhile, it seemed desirable to carry out a few further experiments to determine whether our vaccine possessed any protective properties against the European infectious agent as represented by a strain in our possession—isolated by Breinl and furnished us by the courtesy of Dr. Dyer. This was particularly advisable since in earlier experiments with less potent vaccination materials we found that formalinized *Rickettsia* rarely gave complete protection against the European disease, though often it seemed to exert a partially immunizing effect leading to febrile reactions in the treated animals definitely milder and shorter than those observed in controls.

Three experiments with European virus were done. One was a complete failure, since the dose of virus used for the tests was inactive and failed to infect the controls.

In another experiment—three vaccinated guinea pigs and three controls—there was practically no protection in two of the vaccinated animals, but the third developed a brief and mild febrile period (3 days) with prompt defervescence, as contrasted with severe and typical courses in the controls. This experiment, too, can be summarized as showing little or no evidence of protection.

In the third experiment—again three vaccinated and three control animals—the results were more definitely indicative of some protective effect on the part of the vaccine. The three controls all developed severe and prolonged typhus infections with temperatures rising to above  $104^{\circ}$  on the 8th and 9th days and lasting at approximately this level for 11 to 12 days, accompanied by considerable emaciation of the animals. Of the three vaccinated guinea pigs, one seemed completely protected and the other two had brief and mild fever curves distinctly in contrast to those of the controls, lasting for 3 days above  $104^{\circ}$ . It is worth noting that this experiment was done 107 days after the last administration of vaccine.

#### DISCUSSION

The experiments recorded above leave no question in our minds that active immunization against the Mexican and endemic American varieties of experimental typhus fever can be carried out with killed and washed vaccines prepared by the X-ray rat method described by us. Whether the method will prove effectual in man remains to be seen, but this problem is being actively investigated by Mexican students of the disease—particularly by Varela, by Casco and by Bustamente. That the vaccine, properly controlled, does no injury has been adequately demonstrated by the fact that these workers have vaccinated many thousand individuals without accident.

We have no data to offer yet as to the lasting qualities of the vaccines, nor do we know whether formalin or phenol (which is used by Weigl for his louse vaccines) is preferable as a preservative. That the protection conveyed by the vaccine is not a very short-lived one is indicated by the fact that in experiments carried out from 36 to 107 days after the last vaccination the results were favorable. If there should still be a lingering doubt in the minds of some investigators as to whether or not the *Rickettsiae* obtained by us from the



tunica vaginalis of guinea pigs represent the true etiological agents of the New World disease, we may point to Experiment II, in which the *Rickettsia* vaccine protected against inoculations with the blood of animals at the height of the disease. To assume in that case that the vaccine did not contain the specific agent would necessitate the corollary that the diseases in the Mexican and North American guinea pigs were not typhus fever at all, but a *Rickettsia* disease quite distinct from European typhus.

Since these experiments, together with our previous ones and those of Kemp and of Dyer and his associates, establish the possibility of active immunization with killed *Rickettsiae*, the ideal method to be aimed at should be the development of cultural methods, either on special media or on tissue, which will yield sufficient quantities of *Rickettsiae* for vaccine production. Until this is achieved, we believe our X-ray rat technique to be the most practically applicable method.

In regard to the European variety of the disease, our results remain partial. In view of the results of Varela and his collaborators, who protected with our vaccines against the Tunisian virus, it may be of value to attempt human vaccination with our materials in Africa as it is being carried out in Mexico. We should hesitate to advise such a procedure in Europe until we have achieved more than the partial experimental successes which we have described. Our results in this regard are definitely inferior to most of those reported in guinea pigs with the Weigl louse vaccine. Nevertheless, they are distinctly encouraging to further effort.

#### SUMMARY

1. Vaccines consisting of formalinized *Rickettsiae* of Mexican typhus fever, obtained by our X-ray rat method, produce definite resistance in guinea pigs to subsequent infection with the virus of this disease.
2. The resistance so produced amounts to complete immunity when the subsequent infectious dose is moderate—that is, consists of typhus blood or of tunica material in reasonable amounts (not more than one-quarter of a tunica—*i.e.*, roughly 100 to 250 infectious doses). When, as in the first experiment, excessive doses of infectious material were given, the vaccination protection was, in two of the three animals, incomplete.

3. Subcutaneous vaccination is fully as effective as intraperitoneal—even when the subsequent infection is intraperitoneal.

4. As in previously reported experiments, the vaccines made with the Mexican organisms conferred only partial and feeble protection against the European virus (Breinl strain).

#### REFERENCES

1. Zinsser, H., and Castaneda, M. R., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 840.
2. Weigl, R., cited from Otto, R., and Munter, H., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Microorganismen*, Jena, Gustav Fischer, 3rd edition (Kolle, W., Kraus, R., and Uhlenhuth, P.) 1930, **8**, Liefg. 44, 1193.
3. Dyer, R. E., Workman, W. G., Rumreich, A., and Badger, L. F., *Pub. Health Rep., U. S. P. H. S.*, 1932, **47**, 1329.
4. Kemp, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 353.
5. Varela, G., Parada, A., and Ramos, V., *Proc. Soc. Exp. Biol. and Med.*, 1932, **30**, 206.