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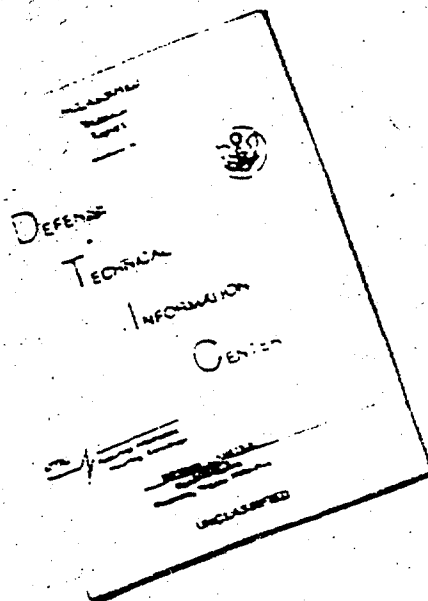
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## TYPHUS FEVER STUDIES

### I. TOXIN IN RICKETTSIA-EGG CULTURES (RICKETTSIA MOOSERI)

Following is a translation of an article by E. Gildermeister and E. Haagen of the Robert Koch Institute, Berlin, which appeared in the German-language periodical Deutsche Medizinische Wochenschrift (German Medical Weekly), Vol 66, 1940, page 878-880.

In the fight against typhus fever, elimination of lice is and will continue to be the most important factor. Only where the populace are infested with lice can typhus fever occur either endemically or epidemically. As a result, eradication of the louse is equal to suppression of typhus fever. If typhus fever is introduced in a people who are free of lice, the sickness generally is limited to a few cases only if defensive measures are taken promptly.

Even immunization against typhus fever, which has made great advances during the past two years, cannot substitute for elimination of the louse in the fight against this disease. First of all, immunization does not provide absolute protection and secondly the production of vaccine for millions of people is not yet possible. We must therefore limit immunizations to those persons who are especially endangered by virtue of their activity; these are doctors, medical assistant personnel, police personnel, etc.

When we were faced with the necessity of producing typhus fever vaccine, it was clear to us that the method of Weigl — from the intestine of infected lice — could not come into the question because of its complicated nature. The Weigl vaccine is the best available at this time, but numerous people who are immune to typhus fever are required for feeding of the infected lice, and they are not available in

Berlin. We therefore decided upon a process which has been practiced in Germany for a long time by Otto and Wohlrab and has been tested under practical conditions. The causative agent of the classic typhus fever, the rickettsia prowazeki, is not used in this method, but rather that of murine typhus fever, the rickettsia mooseri. Due to the close relationship of the two types of rickettsia, one can perhaps expect a vaccine made with rickettsia mooseri to be effective against the classic typhus fever.

Such vaccines can be obtained in three different ways:

1. From rickettsia which have multiplied in the peritoneum of mice,
2. From rickettsia which have been bred in cell cultures according to the method of Nigg and Landsteiner, and
3. From rickettsia obtained from incubated chicken eggs via the Dottersack method of Cox /Dottersack = vitelline membrane, the membrane surrounding the yolk of the egg/

Otto and Wohlrab have used the later method, for the most part, for some time. It is relatively easy to carry out and generally yields an ample result. We therefore decided to use this process ourselves. Privy Counselor /Geheimrat/ Otto and Dr. Wohlrab donated a strain of rickettsia mooseri which had been held in mice to our efforts, for which we are grateful.

The continuous breeding of rickettsia mooseri in white mice is very easy. The brain of typically ill mice is well ground in a mortar and mixed with 10 cc Ringer's solution. 0.25-0.5 cc of this brain suspension are injected into new animals intraperitoneal. The infected animals usually become sick on the 4th or 5th day and die about the 6th or 7th day. Rickettsia can then be found in more or less large amounts in peritoneal smear slides.

The brain of mice thus infected and made sick is then used to breed rickettsia in eggs which have been incubated for 7 days. 0.2 cc of the above described brain suspension is injected into the blunt pole of the egg by means of a syringe. The shell of the egg is first sterilized with iodine and a hole is made with a sterile needle. The hollow needle of the syringe is then inserted horizontally through this hole as far as possible and the contents of the syringe injected.

The eggs infected in this way are then incubated 6 more days and then opened by cutting off the blunt pole with a shears. The embryo is thereupon removed and the remainder of the contents of the egg is poured

into a sterile double dish. The vitelline membrane is then separated from the yolk by twisting around two sterile needles and placed into a sterile dish. Smear slides are then made from the vitelline membrane for examination for rickettsia and aerobic and anaerobic sterilization measures taken, that is to say, it is checked for the presence of bacteria. Only those vitelline membranes which contain rickettsia and are free of bacteria are processed further.

The production of vaccine takes place in the following manner: Each vitelline membrane is put into a sterile glass bottle with glass balls, 25 cc Ringer's solution added; shake the bottle for one hour on the vibrator machine. After material for further egg passage is removed, there follows an addition of 0.5% Formol. After this, shake for 2-3 days with the vibrator, then filter through gauze in order to take out the remnants of the vitelline membrane, thicken to one half volume in the exsiccator with suction pump and then fill to make the original volume with Ringer's solution. The vaccine is now filled into tubes and placed in water bath at 60°C for one hour on two successive days. This completes the production of the vaccine. It is best stored under refrigeration at + 2-4°C.

In the course of this work we made observations which seem to us to be of basic importance and which will be reported below. (Note: In our work with typhus fever we were helped by our technical assistants, Miss Irmgard Ahlfelt and Miss Brigitte Crodel.)

As a means of determining whether the rickettsia-egg cultures which we had started retained their pathogenity for the white mice, a vitelline membrane which had been shown to have rickettsia in the microscopic examination of a smear slide was shaken with Ringer's solution and amounts of 0.5 and 1 cc were injected into white mice i.p. (intra-peritoneal). To our surprise, all of the animals died within 4-20 hours, some of them with cramps. Since the vitelline membranes were definitely determined to be free of bacteria, a bacterial effect could be discounted. Now one possibility was that the vitelline membrane of incubated chicken eggs was poisonous for mice. To clarify this point, chicken eggs which were incubated for 7 days were injected with 0.2 cc Ringer's solution instead of with rickettsia and incubated for six days more. The vitelline membrane was removed in the usual way and shaken for one hour with 25 cc Ringer's solution, then injected i.p. in amounts of 0.5-1.0 cc into white mice, but it showed itself to be completely free of poison; the animals remained alive and showed no signs of sickness.

Further experimentation showed that every rickettsia-positive vitelline membrane suspension from an incubated chicken egg killed mice which had i.p. injections within 24, or at most 48, hours. Since it was injected i.p. in amounts of up to .25 cc or less, less often .1 cc, the poison contained in these vitelline membranes cannot be considered

to be highly effective [Note: meaning unclear in original]. The suspension kills only in exceptional cases when injected sub-cutaneously. The intracerebral method is likewise not dependable.

Guinea pigs given i.p. injections of 1 cc of the rickettsia-vitelline membrane suspension show no ill effects.

Vitelline membrane suspensions from eggs which have been infected with rickettsia and incubated, but which show no rickettsia under microscopic examination — in which, therefore, the infection did not take — also do not contain poison.

As regards the time when the poison developed in infected eggs, our experiments showed that after two days following the infection the vitelline membrane of the incubated eggs did not contain the poison, but from the 4th day on they regularly did contain poison. Rickettsia were also not to be found after two days, but after four days they were always found in amounts equal to those found after six days.

It is emphasized once more that there is no possibility that the poison came from any type of bacteria which happened to be in the eggs, since only those vitelline membranes which were free of bacteria were used in the tests for poison. Furthermore, the mice killed by the poison were free of bacteria in their heart blood.

At this point, it was necessary to determine other characteristics of this rickettsia poison. The experiments made in this connection showed that it is exceptionally labile. The effectiveness of the poison was destroyed by nothing more than keeping the rickettsia-vitelline membrane suspension under refrigeration of + 2°C for seven days. The poison is also rapidly made ineffective through addition of Formol or by heating to 60°C. Thus the possibility of working with a poison which contains no rickettsia is eliminated.

In order to clearly establish the specificity of the rickettsia which we had demonstrated, neutralization tests were conducted with serums of varying extraction. Serums from persons who had been immunized with Otto-Wohlrab vaccine and others immunized with Weigl vaccine were tried. In addition, serum from one of our colleagues who had become infected during her work with egg cultures and suffered a mild illness was available (Weil-Felix after immunization with Otto-Wohlrab vaccine 1:50 +, after the illness 1:1600). Further, there were three serums from persons who had recently survived the classic typhus fever (rickettsia prowazekii), for which we have Dr. Kunert from the Hygienic Institute in Litzmannstadt to thank. Serums from co-workers who had not been immunized served as control serums, also normal rabbit serums. For general interest we also used serum from retroplacental blood in the form of Homoseran (Anhal Serum Institute), which has shown a richness

in protective material of various types (measels and poliomyelitis, for instance).

The tests were designed as follows:

To 1 cc of the rickettsia-vitelline membrane-Ringer's solution, equal or lesser amounts of the serum were added. If a lesser amount of serum was used, Ringer's solution was added in order to bring the volume up to 2 cc. The mixture was left at room temperature for 4-5 hours, then refrigerated until the next day at + 2°C. Then 1 cc of the mixture, which contained 0.5 cc of the rickettsia-vitelline membrane-Ringer's solution, was injected i.p. into each of two mice.

The questions which these tests were intended to answer were the following: 1. does immunity serum neutralize the rickettsia poison? 2. can the rickettsia also be rendered harmless by the serum? The answers to these two questions are given in the following two tables.

TABLE 1

Type and origin of serum	Amount of serum	Ringer's solution	Amount of rickettsia-vitelline membrane-Ringer's solution in cc	Number of mice	Number which died		Still living after 10 days
					within 24-48 hours	later	
Miss C, following laboratory infection with rickettsia Mooseri	0.5	—	0.5	3	—	1	2
	0.2	0.3	0.5	3	—	3	—
Miss A, immunized with Otto-Wohlrab vaccine	0.5	—	0.5	3	1	2	—
	0.2	0.3	0.5	3	2	1	—
Miss H, immunized with Weigl vaccine	0.5	—	0.5	3	2	—	1
	0.2	0.3	0.5	3	1	2	—
Serum from retro-placental blood (Homoseran)	0.5	—	0.5	3	2	1	—
	0.2	0.3	0.5	3	3	—	—
Normal human serum 1	0.5	—	0.5	3	2	1	—
	0.2	0.3	0.5	3	3	—	—
Normal human serum 2	0.5	—	0.5	3	2	1	—
	0.2	0.3	0.5	3	3	—	—



T A B L E 2

Type and origin of serum	Amount of serum	Ringer's solution	Amount of rickettsia-vitelline membrane-Ringer's solution in cc	Number of mice	Number which died		Still living after 10 days
					within 24-48 hours	later	
Miss C, following laboratory infection with rickettsia mooseri	0.5	—	0.5	2	—	—	2
	0.25	0.25	0.5	2	—	—	2
Typhus fever serum Sl.	0.5	—	0.5	2	—	—	2
	0.25	0.25	0.5	2	—	—	2
Typhus fever serum Sk	0.5	—	0.5	2	—	—	2
	0.25	0.25	0.5	2	—	—	2
G, immunized with Otto-Wohlrab vaccine	0.5	—	0.5	2	—	2	—
	0.25	0.25	0.5	2	—	2	—
Serum from retro-pacental blood (Homoseran)	0.5	—	0.5	2	—	—	2
	0.25	0.25	0.5	2	—	—	2
Normal rabbit serum 1	0.5	—	0.5	2	2	—	—
	0.25	0.25	0.5	2	1	1	—
Normal rabbit serum 2	0.5	—	0.5	2	2	—	—
	0.25	0.25	0.5	2	2	—	—
Normal human serum 3	0.5	—	0.5	2	1	1	—
	0.25	0.25	0.5	2	1	1	—
Typhus fever serum R	0.5	—	0.5	2	1	—	1
	0.25	0.25	0.5	2	—	1	1
Control	—	0.5	0.5	2	2	—	—
	—	0.5	0.5	2	2	—	—

From these tables we see the followings:

1. Effect upon the poison: Both the typhus fever serum from Miss C. (infection with rickettsia mooseri) and the typhus fever serum

(rickettsia prowazekii) Sl. and Sk. neutralized, when in large enough doses, the rickettsia poison very definitely, the typhus fever serum R with only one exception. The serum of the immunized persons makes the poison ineffective for the wide majority of the animals, while normal human serum and normal rabbit serum have the opposite effect for the most part. The serum obtained from retroplacental blood showed itself to be particularly effective; the poison was made ineffective in almost all cases.

2. Effect upon the rickettsia: Here, too, the typhus fever serums proved to be effective. In a dose of 0.5 cc they most often prevented the take of the infection. The serum of immunized humans was not able to do this except in one case. Likewise, the few mice which were not protected from the poison effect did not die. Especially noteworthy is the fact that Homoseran protected the mice against typhus fever in the same way — with sufficiently large dosage — as did the typhus fever serums.

We do not want to fail to mention the fact that, for passage breeding of rickettsia in incubated eggs, the vitelline membranes with the greatest concentration of rickettsia are not best suited, for the chicken embryos injected with such material almost all die prematurely; therefore the continuation of the experiment succeeds best when the medium rickettsia content membranes are used. This fact which we discovered shortly after the start of our tests found its explanation with the demonstration of rickettsia poison. It was shown that the poisonous effect was greater when the vitelline membrane was rich with rickettsia. It can then be assumed that the chicken embryo can withstand a certain dosage of rickettsia poison, but as soon as this is exceeded, the embryo dies.

Our experiments clearly proved the existence of a rickettsia poison. Here it is not to be further explained as to what type of poison it is, whether it is an exotoxin or an endotoxin. Based upon our determinations, we are of the opinion that it may well be an endotoxin. Further tests are necessary to decide this question. In any event, it can now be stated that the main symptoms of a clinical case of typhus fever — the exanthema of the skin and the disturbance of the circulatory organs and the central nervous system — are based upon the effect of poison formed by the rickettsia.

Our studies are of course not yet finished, but rather they will be extended, especially in an effort to determine whether that while we learned about rickettsia mooseri also applies to rickettsia prowazekii. This appears to be very likely to us. Another area to be checked is whether rickettsia poison can be found in tissue cultures in the same way as in egg cultures. Such tests have already been started.

In that the poison of the rickettsia mooseri, as was shown, is

very viable, any attempt to kill the rickettsia also makes the poison ineffective. For this reason, it is not now possible to inoculate animals with pure poison without the presence of live rickettsia. Nevertheless, such immunizations should be conducted — with poison and living rickettsia — in order to determine any difference between such immunity serums and those produced from killed rickettsia.

Summary:

1. Confirming the statements of Cox and of Otto and Wohlrab, it was found that rickettsia mooseri, the causative agent of murine typhus fever, could be bred without difficulty in incubated chicken eggs.
2. A poison was demonstrated to be in the rickettsia-containing vitelline membranes of such egg cultures, which, according to the above authors, are used in the production of typhus fever vaccines. This poison kills mice with 24, or at most 48 hours following intraperitoneal injection. This is the first demonstration of a toxin in rickettsia cultures.
3. The facility with which the rickettsia toxin in the vitelline membranes can be demonstrated increases in direct proportion to the increase in the amount of rickettsia present.
4. This toxin is very easily destroyed; it becomes ineffective through the addition of Formalin or by heating to 60°C, or after being stored for seven days. The resistance of the toxin, therefore, is no greater than that of the rickettsia.
5. Typhus fever serums of the murine as well as of the classic typhus fever neutralize the poison. This can also sometimes be done with serums from persons who have been immunized with vaccines made from rickettsia mooseri or rickettsia prowazekii. Serum from normal humans or rabbits does not neutralize the poison. It is noteworthy that serum obtained from retroplacental blood (Homoseran) promptly neutralizes the poison.
6. Typhus fever serums and Homoseran almost always protect mice from sickness following infection with rickettsia mooseri. Serums from immunized persons does this only rarely.
7. The demonstrated rickettsia toxin is apparently an endotoxin.
8. The characteristic symptoms of a clinical case of typhus fever — exanthema, disturbance of the circulatory organs and the central nervous system — may be due to a toxin effect.

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