THE CULTIVATION OF MEXICAN AND EUROPEAN TYPHUS RICKETTSIAE IN THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK EMBRYO *

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In the course of studies on typhus fever continued in this laboratory efforts have been made to compare the biological and serological properties of the Mexican and the European virus strains. While the two are beyond question closely related, determinable immunological differences have recently and clearly been brought out in the vaccination and passive immunization experiments and in the serological reactions described by Zinsser and Castaneda.[1] The most troublesome difference, however, has been the fact that it has not been possible to obtain as extraordinary an accumulation of *Rickettsiae* with the European strain by the rat X-ray method as was possible with the Mexican strain, as a practical method in vaccine production. In attempting to gain more insight into the existing differences a number of experiments have been carried out in this laboratory, the chief purpose of which was to study the two varieties of *Rickettsiae* against the same biological background, other than the louse intestine in which they appear and behave entirely alike. The following experiments would not have been performed had it not been for a casual visit to this laboratory of Dr. Ernest Goodpasture, who described to us in detail his cultivation of a variety of ultramicroscopic agents by the "fertile egg" method, details of which have since appeared in a number of publications from his department.[2, 3, 4] We take this opportunity of listing him, in this manner, as a co-author. We made no changes in the technique that he described, except in point of time and temperature of incubation — matters deemed advisable in view of the experience with *Rickettsiae* gained here. The technique in brief, then, is as follows.

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METHOD

Fertile hen's eggs were incubated at 37.5° to 38° C for 8 to 9 days. At the end of this time the eggs were washed with alcohol and flamed. Windows 0.5 to 1 cm. square were cut in the shell by means of a razor blade. In most instances the inside acellular shell membrane was not injured. Hot paraffin was allowed to flow over this layer, which was then opened by cutting around the edges of the window with a pair of fine scissors. A small amount of emulsified tunica

TABLE I

Summary of Results of Cultivation of Rickettsiae in the Chorio-Allantoic Membrane of the Chick Embryo

Strain	Inoculum	Total number of eggs inoculated	No. of eggs with dead embryos	No. of eggs with living embryo	
				Positive for *Rickettsiae*	Negative for *Rickettsiae*
Mexican *Rickettsiae*	Tunica	23	13	4	6
	Spleen	10	1	1*	8
	Egg	15	9	4*	2
European *Rickettsiae*	Brain	13	9	0	4
	Spleen	12	7	2	3
	Brain and spleen	9	7	1	1

* One each by smears only.

exudate or brain and spleen material was dropped with a capillary pipette on the extra-embryonic membrane, the chorion being uppermost. A sterile coverslip was placed over the opening and it was sealed with hot paraffin. The eggs were then reincubated at 33° C and opened after 7 to 10 days for examination. Smears were stained with Castaneda's methylene blue - safranin stain,[5] and tissue fixed in Regaud's solution (potassium bichromate 2.5 gm., sodium sulphate 1 gm., water 100 cc., to which is added 20 cc. of formalin immediately before use) and later stained by Giemsa's method. In several instances the material was also inoculated into guinea pigs and a typical response was obtained in these animals. Immunity tests showed them to be protected from subsequent homologous infections. Cultures for bacteria were made from the eggs in which no obvious signs of contamination could be observed, and in only four instances was there any growth on blood agar plates.

Results

The results of these experiments are summarized in Table I. In general it was easier to infect eggs with Mexican *Rickettsiae* and these appeared greater in number. Many embryos were found dead a few days after inoculation. This was particularly true with the European *Rickettsiae*. Even discounting the eggs with dead or autolyzed embryos, in which we never found positive results, the percentage of positive findings was so low and the amount of virus obtained so scarce that it was impossible to make vaccine from them. However, the microscopic appearance of the infected chorio-allantoic membrane seems to be of sufficient additional interest and this is, therefore, briefly described.

Details of Representative Experiments

1. Experiments with Mexican Rickettsiae: Eggs were received October 21, 1932, and kept at 37.5° C until October 31st. On this day three eggs inoculated with emulsified tunica material from Guinea pig 365 showed typical lesions with *Rickettsiae*.

Egg 1 kept at 33° C and opened November 3rd was negative. Egg 2 kept at 38° C and opened November 4th was negative. Egg 3 kept at 33° C and opened November 8th showed a thickened membrane over the exposed area, not adherent to the shell, but there were no signs of bacterial infection. The embryo was alive. A smear showed but few *Rickettsiae*. Part of the membrane was inoculated into Guinea pig 372, which showed slight fever but typical tunica swelling. The same animal inoculated again with tunica material from a Mexican typhus guinea pig showed complete immunity. Cultures from the egg on blood agar plates were sterile. The main part of the infected membrane was fixed in Regaud's solution and sections were cut and stained with Giemsa. Figure 1 is a drawing made from a microscopic field of this membrane under a magnification of 600 diameters. It will be seen that there are *Rickettsiae* crowded in some of the cells of the ectodermal layer which, in morphology, staining reaction with Giemsa, and in their intracellular positions, are indistinguishable from the same organisms seen in the tunica cells of guinea pigs or in the intestinal cells of human lice, polyplax or fleas.

2. Experiments with European Rickettsiae: Eggs were received November 18th and kept at 38° C. Three eggs were inoculated on November 22nd with material from Guinea pig 377, suffering at the time from a typical attack of European typhus infection. The temperature of the guinea pig was 106° F when it was sacrificed, 9 days after intraperitoneal inoculation. There was no scrotal swelling at the time.

Egg 1 was inoculated with brain, Egg 2 with brain and spleen, and Egg 3 with spleen only. It is important to note that, as usual, no *Rickettsiae* could be found in the tissue material inoculated.

On November 29th Eggs 1 and 2 were opened. In Egg 1 we had negative results. There apparently was no change — the membranes were not thickened and smears and sections were both negative for *Rickettsiae*. Egg 2, inoculated with brain and spleen, showed slight thickening of the membrane, and on smear a few intracellular and extracellular *Rickettsiae* were found after prolonged search. This membrane was cultured on blood agar with negative results. Sections fixed in Regaud's solution and stained with Giemsa showed a fairly large number of cells containing typical *Rickettsiae*.

Egg 3 opened on December 2nd was negative. Figure 2 represents a drawing of cells containing the organisms. In morphology, in staining reaction to Giemsa and in intracellular grouping these organisms were identical with *Rickettsiae* as seen in the cells of louse intestine infected with European typhus, and appeared to be identical with the Mexican *Rickettsiae* in the other eggs, except that perhaps they were in average measurement slightly smaller.

A part of the membrane from Egg 2 was inoculated into Guinea pig 390. This animal showed a typical temperature curve, reaching 105° F and above on the 8th and 9th day, rising to 106° F on the 10th and 11th day, and on the 12th day, when the temperature was 105.5° F, the animal was killed for histological examination and for inoculation. Guinea pigs 399 and 400 were inoculated with brain and blood from this animal and later both showed typical passage strain reactions. The brains of both Guinea pigs 390 and 399 showed characteristic brain lesions in considerable profusion. These have been independently checked by a number of experienced observers. The absence of scrotal swelling, indeed, as well as the profusion of the brain lesions, characterizes it without doubt to be of the European type of infection. This is further borne out by

the immunity test. Guinea pigs of the third, fourth, and fifth generations (Nos. 414, 418 and 443) were reinoculated, after 6 weeks to 2 months, with material from European typhus guinea pigs, and all three were completely immune.

3. Experiments with Subcultures in Eggs: We were interested to see if the amount of *Rickettsiae* in these membranes might not be increased by repeated transfer from egg to egg. One of these experiments is as follows.

On January 19th four eggs were inoculated with tunica material from Mexican typhus Guinea pig 447, smears from which showed *Rickettsiae*. Egg 1 opened January 28th showed a living embryo with a thickened membrane. Blood agar plate cultures were sterile. Both smear and section were positive for *Rickettsiae*. The material was ground in a mortar and inoculated into four new eggs. Among these, Eggs 6 and 7 were autolyzed, the other two showing positive results. Material from one of these, Egg 8, was inoculated into another series of four eggs, with positive results in one. While there was suggestive evidence of an increase in the number of *Rickettsiae* in the eggs of the second and third generations, this did not appear to be of sufficient degree to warrant further transfer.

Histological Examination

Changes Observed 8 to 9 Days after Infection: The appearance of the normal chorio-allantoic membrane from hatching chicks has been described by Woodruff and Goodpasture.[2] It consists of a thin layer of reticulated mesothelial tissue lined by one or two celled layers of ectodermal and endodermal tissue. At this stage of development the ectodermal layer is often absent. A great change occurs when infection with *Rickettsiae* takes place. The whole membrane with all three layers is much thickened. This naturally varies with the degree of infection. In the lightly infected membranes solitary thickening of only the ectodermal lining takes place. Usually, however, the number of cellular elements in the mesothelial layer is found to be increased. In a case of average severity the ectodermal lining is about ten cells thick, covered on the outside with a layer of degenerated cells. The endodermal lining is slightly thickened. There is a great increase in the cells with a deeply stained, round nucleus in the mesothelial

layer. These often form clumps or nodules of various sizes. Most of the cellular elements in these nodules are far too degenerated to be differentiated. A large number of cells containing coarse eosinophilic granules also appear grouped with the mononuclear cells. Often in the center of these nodules small blood vessels containing red blood cells can be distinguished. Figure 3 is a photomicrograph showing one of these nodules, together with the increased cellular element in the surrounding areas. It is interesting to note that typhus infection of the chorio-allantoic membrane gives an entirely different picture from that infected with vaccinia or fowl pox. In fact, when the organisms are few the presence of these changes often encourages prolonged search and frequently leads to subsequent finding of the organisms.

Distribution of Organisms in the Infected Chorio-Allantoic Membrane: By far the majority of the *Rickettsiae* are found in the degenerated outer layer of the ectodermal lining. There they are mostly intracellular and often in large clumps. A few are also found in the tissue spaces and these often assume a much elongated form. Pinkerton [6] considers these the most actively growing *Rickettsiae*, as shown in his tissue culture experiments. When the infection is heavy a few *Rickettsiae* may be found in some of these nodules. How they reach there it is difficult to determine. In one or two sections we have observed them along the wall of a small capillary, but we have not succeeded in demonstrating them in the endothelial cells lining these vessels. One may perhaps speculate about the formation of these nodules as starting from tissue reaction to local deposit of organisms and their metabolic products, with subsequent death of these cells due to impairment of blood supply.

Discussion

While few students of typhus fever have any doubts concerning the etiological importance of *Rickettsiae*, both in the European and the Mexican infections, there still arise occasional questions regarding the significance of the organisms found by Mooser in the tunica lesions. While we believe that the work of Mooser, as well as the extensive experimental cross-indexing of the facts bearing upon this point undertaken in this laboratory, has removed all possibility of error, every additional point of evidence is of value in so important a question.

The fact that the eggs inoculated with the Mexican tunica material develop morphologically typical *Rickettsiae* and that attempts to cultivate bacteria from these eggs are unsuccessful, added to the characteristic results of inoculation of the egg material into animals, brings further evidence to the array of proof already submitted.

In regard to the inoculations of the eggs with European material the results indicate that from tissue material in which *Rickettsiae* are apparently too few to be found by smear a culture can be produced in which they are plentifully apparent and from which the disease can again be propagated. The similarity of these cultivated *Rickettsiae* to those found in the European lice adds, we think, more strength to the assumption of the etiological significance of the *Rickettsia prowazeki*.

Furthermore, it has been of exceptional interest to us that by identical methods the European and the Mexican *Rickettsiae* can be studied against the same biological background and proved to be indistinguishable in their behavior under the same cultural conditions. This is a direct demonstration of the close similarity between the two organisms and should assist materially in removing any lingering doubt as to whether Mooser's organism may have been picked up in experimental animals during inoculation passage or not. This question was raised not long ago and every point of evidence that can clarify it is of more than ordinary importance because of the extensive endeavors to produce prophylactic vaccines and potent antityphus sera with the Mexican tunica organisms.

The fact that the inoculation of guinea pigs from the European and Mexican typhus infected eggs, respectively, has produced the two characteristic types of the disease is another point of evidence that, in spite of their close similarities, the two organisms are not absolutely identical. Though possibly derived from the same original stock, adaptation in passage through rodents, fleas and polyplax may be the cause of slight biological modifications in the Mexican strain.

Our results in the egg method of cultivation so far have not given much hope that we may obtain a sufficient number of *Rickettsiae* in this way. Several egg to egg transfers have not added materially to the yield. However, the suggestive evidence of these interesting histological appearances may perhaps throw some light on the formation of the typhus nodules in man and in experimental animals.

SUMMARY

It has been found that both Mexican and European typhus *Rickettsiae* are able to infect the chorio-allantoic membrane of the chick embryo, although the results do not lead us to hope for its practical use in the production of vaccines. The interesting histological appearance of the typhus-infected membrane and the distribution of *Rickettsiae* are briefly described. Possible significance of this finding to clarify further the relation between *Rickettsia prowazeki* and Mooser's bodies is discussed.

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DESCRIPTION OF PLATE

PLATE 70

FIG. 1. Drawing of a Giemsa-stained paraffin section of egg inoculated with tunica material from a Mexican typhus guinea pig showing *Rickettsiae* of the Mooser type.

FIG. 2. Drawing of a Giemsa-stained paraffin section of embryonic membrane from an egg inoculated with spleen and brain tissue from a guinea pig infected with European typhus of the Breinl strain, and showing *Rickettsiae prowazeki*.

FIG. 3. Photomicrograph showing one of the nodules in the mesothelial layer. × 600.