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CULTIVATION OF RICKETTSIAE OF THE ROCKY MOUNTAIN SPOTTED FEVER, TYPHUS AND Q FEVER GROUPS IN THE EMBRYONIC TISSUES OF DEVELOPING CHICKS[1]

By Dr. HERALD R. COX

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I AM deeply conscious of the honor which the American Association for the Advancement of Science has conferred in selecting me as the recipient of the Theobald Smith award.

I take this opportunity to express my appreciation to my former chief, Dr. Peter K. Olitsky, of the Rockefeller Institute, for his continued and neverfailing interest in my work, to Drs. R. R. Parker, director of the Rocky Mountain Laboratory, and R. E. Dyer, chief of the Division of Infectious Diseases, The National Institute of Health, for their sympathetic understanding and cooperation in making these studies possible, and to my assistants, E. John Bell and Lyndahl E. Hughes, for their loyal and invaluable aid.

In 1938 the author reported: First, a simple tech-

[1] Contribution from the Rocky Mountain Laboratory (Hamilton, Montana) of the Division of Infectious Diseases of the National Institute of Health. Address delivered on September 22, 1941, to the Section on Medical Sciences upon receipt of the Theobald Smith Award of the American Association for the Advancement of Science.

nique by which the rickettsiae of the Rocky Mountain spotted fever and typhus groups can be cultivated abundantly in the yolk sac of the developing chick embryo; and secondly, that suspensions of yolk sacs infected with spotted fever or typhus rickettsiae are from 100 to 1,000 times more infective than other tissues of the developing chick embryo or mammalian tissues.[2] Later it was shown that yolk sac cultures were highly suitable for the production of consistently good immunizing vaccines for Rocky Mountain spotted fever, European and endemic typhus and American "Q" fever.[3, 4, 5, 6, 7] In the following pages the present method for initiating and maintaining passage strains in eggs will be described and the results of the use of the yolk sac technique for cultivating rickettsiae and preparing rickettsial vaccines brought up to date.

METHOD OF INOCULATING EGGS AND MAINTAINING STRAINS

Briefly, this is as follows: Fertile eggs incubated for 6 to 7 days at 39° C. are injected in the yolk with infectious material by means of a hypodermic syringe and a 20-gauge needle, 1 to 1¼ inches long. The inoculum, consisting of 0.5 to 1.0 cc of infected, defibrinated guinea pig blood, testicle washings, spleen or brain is introduced through a needle-sized opening in the air-sac end of the egg. After sealing the hole with paraffin, the inoculated egg is incubated at 32° C. in the case of spotted fever, or at 37° C. for typhus or the other rickettsial infections discussed in this paper. Upon death of the embryo, which usually occurs in 3 to 5 days, depending upon the species of rickettsia used, transfer to other fertile eggs is made by means of 0.5 cc of a 5 to 10 per cent. suspension of yolk sac in a 50–50 mixture of sterile beef infusion broth and saline or by using a like quantity of undiluted yolk fluid. Passage strains can be maintained by either method. Yolk fluid is satisfactory for maintaining strains, but when the tissues are to be used for vaccine production, yolk sac suspension is the preferred inoculum because of its markedly greater infectiveness.

Rickettsiae of the following diseases have thus far been cultivated and studied by means of the above-described technique: Rocky Mountain spotted fever (a western Montana, *Dermacentor andersoni* strain and an Iowa, *Dermacentor variabilis* strain), endemic typhus (Wilmington strain), epidemic or European typhus (Breinl strain), boutonneuse fever (a Moroccan strain), Brazilian spotted fever (São Paulo and Minas Geraes strains), Tobia petechial fever of Colombia, *maculatum* infection (from *Amblyomma maculatum* collected in Texas and Georgia), Australian and American "Q" fever, and South African tick-bite fever.

This method has also been used by others for cultivating rickettsial agents, viruses, bacteria and spirochetes.

When establishing strains in eggs there is a period of adaptation before optimal growth occurs. In the first few passages the embryo does not die for a relatively long period, and maximum multiplication of rickettsiae is usually not achieved before 4 to 6 passages. Strains once established can apparently be maintained indefinitely. As a matter of fact, we have had to rely upon the egg method for the uninterrupted maintenance of certain rickettsial strains such as boutonneuse fever, "maculatum" infection and *variabilis* strains of spotted fever. At Hamilton these can be maintained in guinea pigs either not at all or only with the greatest difficulty.

Guinea pigs inoculated with recently established yolk sac strains of the various rickettsioses studied exhibit the following differences when compared with animals receiving guinea pig passage strains: The incubation is usually shortened to 24 to 48 hours; the fever is higher and the febrile period more prolonged; endemic typhus, European typhus, "maculatum" infection and *D. variabilis* spotted fever infections are sometimes fatal; erythema and swelling of the scrotum is frequent in animals infected with European typhus and *variabilis* spotted fever and is intensified in those ill with boutonneuse fever, endemic typhus, "maculatum" infection and the highly fatal type of spotted fever and its South American relatives; scrotal necrosis and sloughing is often more extensive in those infections in which it commonly occurs and may occasionally be seen in diseases in which otherwise it never appears; and finally, in titrations of highly virulent strains of spotted fever even the end-point infective dilution causes fatal infection and death. However, this quality of increased virulence for guinea pigs does not persist with further guinea pig passage. Instead, the usual strain characteristics are generally regained within 3 or 4 transfers.

After strains are carried through a number of yolk sac passages a rather constant killing time for the embryo is established. Guinea pigs inoculated with concentrated yolk sac suspensions of the later egg passages may still show a shortened incubation period, yet the severity of the course of infection is noticeably lessened, as a rule, and in quantitative tests animals that receive the higher dilutions often suffer only inapparent, immunizing infections. This has been found

[2] Herald R. Cox, *Pub. Health Rep.*, 53: 2241, 1938.
[3] *Ibid.*, *Pub. Health Rep.*, 54: 1070, 1939.
[4] Herald R. Cox and E. John Bell, *Pub. Health Rep.*, 55: 110, 1940.
[5] Herald R. Cox, "Cultivation of Rickettsiae in the Embryonic Tissues of Developing Chicks." Presented before the Sixth Pacific Science Congress, July 29, 1939.
[6] Herald R. Cox and E. John Bell, *Pub. Health Rep.*, 54: 2170, December 8, 1939.
[7] Herald R. Cox, *Am. Jour. Trop. Med.*, 20: 463, 1940.

true for all the rickettsial strains studied, but particularly so for those of American and Australian "Q" fever and the *Dermacentor variabilis* strain of spotted fever.

The features that make the yolk sac technique of particular value are its extreme simplicity and the ease with which cultures may be maintained with a minimal risk of contamination. During the past year we have used approximately 30 dozen eggs daily and have found contaminants in less than 1 egg in 2,500.

PREPARATION AND POTENCY TESTING OF VACCINES

Experience has suggested that it is best to use only organisms of maximum virulence in the preparation of *killed* vaccines. Therefore, our present practice is to use for this purpose only strains that have been carried through a limited number of egg passages. Rocky Mountain spotted fever and typhus strains employed for vaccine manufacture are alternated between a series of 40 to 50 yolk sac passages and several transfers through guinea pigs. Nevertheless, we have produced good vaccines against spotted fever and European typhus with materials from eggs of the 246th and 90th passages, respectively.

Vaccines have been prepared by a variety of methods, too numerous to mention. Several have given good results consistently. We will describe only the one that has been employed in making the bulk of the Rocky Mountain spotted fever and epidemic typhus vaccines used for field trials. While the pooled embryonic tissues were used (yolk sac, chorio-allantois and embryo) for these vaccines, we have ample evidence that much richer vaccines in proportion to residual protein may be prepared from only the yolk sac and chorio-allantois or from yolk sac alone. *Again I wish to emphasize that good vaccines can not be obtained consistently without using the yolk sac.*

Upon death of the embryos (in spotted fever this occurs 2 to 3 days, in typhus 4 to 5 days, after inoculation) the pooled embryonic tissues are harvested from all eggs of the same transfer. These are weighed and homogenized to a 12½ per cent. suspension in saline containing 0.5 per cent. phenol and 0.3 per cent. formalin.[8] This suspension is centrifuged at 5,000 r.p.m. for 50 to 60 minutes and the supernatant fluid, which contains the great bulk of lipoids and some soluble proteins, is poured off. The sediment is resuspended with aid of the homogenizer in a volume of saline equal to the original weight of the pooled tissues. Phenol and formalin are added to give a final concentration of 2.0 per cent. and 0.3 per cent., respectively. The resuspended material is placed at room temperature for 6 to 7 days and shaken vigorously daily. During this interval the great bulk of protein is precipitated by the phenol. The suspension is then diluted with 5 volumes of sterile saline and stored at 36° F. for 7 or more days. It is finally centrifuged at 2,500 to 3,000 r.p.m. for 20 minutes, and the resulting supernatant fluid constitutes the vaccine.

The method of preparation described does not give as great a yield as the one previously reported, but does produce a more potent vaccine with less residual extraneous protein. It is highly practical from the standpoint of cost, ease of manipulation and quantity production. Approximately one liter of vaccine can be prepared from 20 eggs. A bacteriologist and two assistants, provided with proper facilities, can readily prepare from 40 to 50 liters of vaccine per week. At Hamilton we have found it feasible to prepare these vaccines in lots of 25 to 35 liters each.

POTENCY TESTS

Seven guinea pigs are used for testing the potency of each lot of spotted fever vaccine. Each animal receives two ½ cc injections of vaccine subcutaneously 5 to 7 days apart. Fourteen days later each is tested for immunity by injecting intraperitoneally 1 cc of citrated blood taken from infected guinea pigs on the third or fourth day of fever. A suitable number of control guinea pigs receive the same inoculum. Highly virulent strains that kill 80 per cent. or more of the control animals are always used, and repeated tests have shown that 1 cc of such infected blood contains from 100 to 1,000 infectious doses for guinea pigs. Temperatures are taken daily for 10 days, and animals that show temperatures of 39.8° C. or higher for 2 or more consecutive days are considered as having spotted fever unless it is quite obvious that some intercurrent infection is present. Five of the 7 test guinea pigs must show complete protection before the lot of vaccine is considered usable.

Typhus vaccine is tested in much the same manner. Twelve guinea pigs are used for the potency test, and each receives two 1 cc injections of vaccine. The test dose for immunity consists of 1 cc of a lightly centrifuged (1,500 r.p.m. for 10 minutes in an International Size 2, horizontal head centrifuge) 5 per cent. suspension of infected brain tissue taken from guinea pigs on the fourth or fifth day of fever. Repeated tests have shown that this inoculum contains 100 to 1,000 infectious doses. Temperatures are taken for 18 days, and 9 of the 12 vaccinated guinea pigs must show complete protection before the vaccine lot is issued.

Quantitative tests carried out recently with vaccines prepared as described above revealed that guinea pigs can be completely protected against the standard test

[8] We have found the Waring-Blendor unit (manufactured by the Waring Corporation, New York City) to be an invaluable aid for homogenizing large amounts of tissue in minimum time. A 51° angle centrifuge was used in all experiments.

dose of spotted fever blood virus by giving them as little as 1/16 cc of vaccine on two occasions. In other experiments it was found that the standard dose of vaccine used in the spotted fever and typhus tests protected guinea pigs in each instance against 100,000 to 1,000,000 infectious units of yolk sac virus. These results certainly indicate a high degree of protection. However, as previously stated, still more potent vaccines can readily be prepared by simply increasing the relative concentration of yolk sac tissue in the final product.

Antigenicity of Chick Embryonic Tissues

I wish to emphasize that we early recognized the serious problems involving sensitization that might arise through the use of such a vaccine. Animal experiments along these lines can be summarized by stating that guinea pigs injected with amounts of vaccine varying from 0.1 to 1.0 cc proved to be entirely unaffected by a shocking dose of 1 cc of the same material given intracardially 20 to 40 days later. These results suggest that any serious sensitization problem is unlikely.

Data collected in vaccinating the personnel of the Rocky Mountain Laboratory and the results of rather extensive field trials carried out this past year appear to support this premise. The laboratory group receiving chick vaccine consisted of 170 persons, including families of the workers. The number of vaccinations and amounts given ranged from a single injection of ½ cc to 14 doses of 1 cc each. In these tests monovalent spotted fever and typhus vaccines, as well as a bivalent vaccine (spotted fever and epidemic typhus) and a trivalent vaccine (spotted fever, epidemic typhus and American "Q" fever) were used. None of the vaccinated individuals reported any reaction other than a slight local tenderness at the site of inoculation, which always disappeared within a day or so.

In addition, more than 226 liters of typhus vaccine, enough for approximately 75,000 people, and more than 200 liters of spotted fever vaccine, enough for approximately 50,000 people, were used in field trials this past year.

The typhus vaccine was sent to Hungary, Rumania, Spain and China; the greatest amounts to the last two countries. Unfortunately, because of the upset conditions due to the war, the results of these trials have either been inconclusive or have failed to reach us. At the present time an attempt is being made to obtain a thorough test of the typhus vaccine in Bolivia. Drs. R. E. Dyer and N. H. Topping, of the National Institute of Health, are now in Bolivia for this purpose.

Physicians to whom the spotted fever vaccine was sent were requested to make careful observations of all people vaccinated and to immediately notify us of any consequential reaction or the occurrence of a spotted fever infection. No serious reaction has been reported. In fact, the evidence suggests that this vaccine is more readily tolerated than the tick type, and that it can be used for patients who have had to discontinue taking the latter. One report to the contrary was received. Noteworthy is the fact that there were no reactions in a number of persons known to be allergic to egg protein.

One case of spotted fever was reported in a vaccinated individual. The attending physician informed us that the patient, a man 53 years old, showed a typical rash. However, he was only mildly ill for less than 2 weeks; was not hospitalized, and continued to work some each day.

It is too early to attempt to draw any conclusions concerning the immunizing value of these vaccines in man, but the results to date are encouraging.

Other Rickettsial Vaccines

We have used essentially the same method to prepare vaccines against endemic typhus, American "Q" fever, boutonneuse fever and Tobia fever. This last-named disease apparently is a highly virulent form of spotted fever that occurs in limited areas in Colombia, South America. Spotted fever vaccine prepared from highly virulent western Montana strains confers complete protection against Tobia fever, and Tobia fever vaccine similarly confers complete protection against western Montana strains. We recently sent Tobia fever vaccine to Dr. Luis Patiño-Camargo, director of the Federico Lleras Institute, Bogotá, Colombia, who reported that it afforded complete protection to guinea pigs against his strains of Tobia fever. We are now supplying Dr. Patiño with sufficient quantities of vaccine to take care of the needs in the several endemic foci.

Preparation of Rickettsial Suspensions for Agglutination and Diagnostic Skin Tests

The abundant growth of the various strains of rickettsiae in the yolk sac has made it possible to prepare, by fractional centrifugation methods, practically pure suspensions of rickettsiae suitable for agglutination tests. Formalinized suspensions of American and Australian "Q" fever and European typhus rickettsiae have been prepared and are quite stable in storage and quite agglutinable by specific antisera. For over a year we have routinely run agglutination tests for American "Q" fever on all sera sent in for Weil-Felix, *B. tularense* or *B. abortus* tests.

More recently we have prepared partially purified suspensions of Rocky Mountain spotted fever rickettsiae, and studies are now in progress to determine if

these, as well as similar suspensions of European and endemic typhus and American "Q" fever rickettsiae, can be used for diagnostic skin tests.

The method of thus preparing practically pure suspensions of rickettsiae by relatively simple procedures opens up many additional possibilities of study along immunological, serological and chemical lines.

OBSERVATIONS RELATIVE TO A *Dermacentor variabilis* STRAIN OF ROCKY MOUNTAIN SPOTTED FEVER MODIFIED DURING YOLK SAC PASSAGE

In conclusion I would like to report observations relative to a *Dermacentor variabilis* strain of spotted fever that has been maintained in eggs for 240 serial transfers since April, 1938. This strain was originally isolated in guinea pigs by inoculating them with a suspension of tissues from *Dermacentor variabilis* ticks collected in Iowa. Several transfers with spleen tissue were successfully made in guinea pigs, but the infection was very mild and a number of animals showed only inapparent infections or failed to react. The strain in guinea pigs was finally lost, but fortunately had already been established in eggs. Tests carried out with yolk sac suspensions of the eleventh and fifteenth egg passages revealed that a marked change, characterized by much greater virulence for guinea pigs, had taken place. Thirty-six guinea pigs were inoculated intraperitoneally with 1 cc each of a 10 per cent. yolk sac suspension. All had high fevers, prolonged temperature curves, erythema and swelling of the scrotum. Of 19 that showed scrotal necrosis and sloughing, 10 died. Titration tests of this same suspension resulted in frank infections, typical of spotted fever in dilutions up to and including one to a million. No inapparent immunizing infections occurred in those animals inoculated with higher dilutions. This enhanced virulence was maintained through about 50 passages. Tests made at random between the fiftieth and one hundred and twenty-fifth egg passages revealed the yolk sac suspensions were becoming markedly less virulent and that a great number of inapparent, immunizing infections were being induced in inoculated guinea pigs. For the subsequent 100 and more passages this strain has regularly killed chick embryos on the third day after inoculation and stained yolk sac preparations have shown just as many rickettsiae as any of our highly virulent strains, yet guinea pigs inoculated intraperitoneally with as much as 1 cc of a 10 per cent. yolk sac suspension have either failed to show any febrile reaction or at most exhibit a slight temperature rise lasting not more than 1 or 2 days. Animals injected subcutaneously with similar suspensions seldom show any reaction. In fact, we have titrated yolk sac suspensions of this avirulent, *variabilis* strain on numerous occasions and found that inapparent, immunizing infections resulted in guinea pigs receiving dilutions as high as 1 to 100,000. However, *the important finding* is that these animals, even when completely afebrile, are later solidly immune to massive doses of highly virulent strains. Furthermore, identical results have been obtained in rhesus monkeys. Attempts to reestablish this strain in guinea pig passage by transfers of blood, testicular washings and spleen suspensions have thus far failed.

Long-term tests are now under way to determine if the degree of protection afforded by this avirulent strain is as solid and lasting as that produced by killed vaccines. Theoretically, we believe it should be even more so. If this proves true, we may eventually be able to immunize man with modified, living strains of spotted fever virus in much the same way as we now immunize against yellow fever. We already have evidence that European typhus, endemic typhus and American and Australian "Q" fever as well as certain other rickettsial strains may similarly be modified in virulence for mammalian hosts by prolonged maintenance in eggs.

RAFINESQUE'S INTERESTS—A CENTURY LATER: MEDICINAL PLANTS[1]

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To have been selected to participate in this Centennial Memorial to Constantine Rafinesque brings to me feelings of profound humility, on the one hand, those of great satisfaction on the other; humility because the mental stature and achievements of Rafinesque so dwarf those of ordinary men as to make him almost legendary; satisfaction because of the honor so deeply felt in being allowed to become affiliated with this magnificent occasion and because, secondly, it gives me an opportunity of expressing thoughts concerning plant drugs which I have entertained for some time silently. I was indeed happy when Dr. Brown assigned me the subject dealing with some of the problems within the field of medicinal plants.

[1] Part of a symposium during the Rafinesque Centennial Memorial, Transylvania College, Wednesday, October 30, 1940, Lexington, Ky.