Published February 1, 1939

A STUDY OF RICKETTSIAE GROWN ON AGAR TISSUE CULTURES*

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(Received for publication, October 7, 1938)

The main objective of the recent work in this laboratory on typhus fever has been the development of a method by which it might be possible to obtain large amounts of virulent Rickettsia for purposes of vaccine production. It has been relatively easy to obtain enormous yields of the murine type of Rickettsia by the x-ray rat method, a technique, previously discussed (1), which has been in weekly routine use in this laboratory for several years. This method has never, however, been successful with the classical or European strain of typhus virus, a fact which, more than any other, has convinced us that there is a deep seated biological difference between the two closely related strains. We have indicated elsewhere that there is reason to assume that the classical virus may have developed from the murine by centuries of man-louse-man transmission (2).

Since the Weigl method of vaccinating with the phenolized intestinal contents of infected lice is, for technical reasons, inapplicable to large scale immunization, the efforts of a number of investigators turned to tissue culture by the Maitland method as modified by Nigg and Landsteiner (3). This method has been found effective for vaccination purposes both by Kligler and Aschner (4) and by Macchiavello and one of us (5).

After considerable modification of the original Maitland technique, however, we concluded that for practical, large scale vaccination this technique, while feasible, was unnecessarily time-consuming and difficult.

In consequence we sought for better methods of cultivating Rick-

* This work was in part supported by a grant from the Commonwealth Fund.

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ettsia and in the course of these efforts studied, with Schoenbach (6), the physiological conditions prevailing in Maitland cultures, both in regard to the metabolic processes going on both in uninoculated flasks and, after inoculation, with filterable virus agents and with Rickettsiae respectively. These studies showed that the conditions most suitable for Rickettsia cultivation were those in which appropriate tissue cells could be preserved, viable and chemically unchanged, for 10 days or more without too active a metabolism. The method that was developed from these premises seems to have solved our problem and, at the same time, appears to furnish a new principle of tissue cultivation applicable, with minor alterations, for the multiplication of some virus agents. Preliminary communications have been published (7) but the method has been perfected during the last few months and details are now published in order to make it possible for other investigators to make use of it.

The Method

The method depends on furnishing a backlog of isotonic agar partially buffered and adjusted by the addition of Tyrode solution and serum, either of horse or beef, or of any other species appropriate for the work to be done. When living bits of tissue are laid upon this agar, diffusion into the medium supplies conditions for the removal of metabolic products and for the supply of the necessary materials for the maintenance of the cells.

The production of the agar is the most important factor in the success of the technique and is, for this reason, described in detail.

1. A 3 per cent agar solution is made in distilled water. The agar used by us is the Difco granular variety. This agar solution is dissolved at 15 pounds for 15 minutes in the autoclave, then cooled and kept at 45°C. in a water bath.

2. A double strength Tyrode solution is made corresponding in every way to the original formula except that 2 gm. of sodium bicarbonate are used per liter. To 300 cc. of this double strength Tyrode are added 200 cc. of the appropriate serum, in our Rickettsia work horse serum, and 16 cc. of 0.04 solution of phenol red. This Tyrode-serum mixture is filtered through a Seitz filter. We have found it important to wash the Seitz filter disks rapidly with distilled water at least three times in order to diminish alkalinity. These washed disks are dried slowly in the incubator and then sterilized, in the holders, in the autoclave.

The Tyrode-serum mixture, as given above, after filtration, is warmed to 45°C. and mixed at this temperature with 300 cc. of the 3 per cent agar. The mixture

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is then poured into 6 × 1 inch test tubes, with precautions for sterility (use of a sterile siphon system) in 12 cc. amounts. The tubes are slanted and left at room temperature to harden. Then they are stoppered with sterile No. 4 stoppers. The final reaction should be about 7.8. The slants should be kept upright at room temperature for 2 or 3 days so that the water of condensation may sink to the bottom to be removed with a capillary pipette before the tubes are planted, since excessive moisture interferes with growth. They are kept at room temperature for an additional check on possible contamination and after this can be kept in the ice chest until used.

The tissue at first used for Rickettsia cultivation was guinea pig tunica of the testicle. We have found that it is quite easy to start new Rickettsia cultures directly on this medium instead of first passing them through a series of Maitland flasks. For such isolation, the tunica is removed from animals infected with passage virus; in the case of the murine strain at the first indication of scrotal swelling, in the case of the classical strain irrespective of swelling on the 6th or 7th day after intraperitoneal infection. In the case of Rocky Mountain spotted fever strains similar isolation may be practised and has succeeded in the hands of Pinkerton for the Western spotted fever strain and in those of one of us with a strain of spotted fever isolated from a case occurring on Cape Cod. In these cases the tissue used for first isolation consisted of infected spleen as well as of tunica. The tunica vaginalis is removed under sterile precautions, is finely minced in a test tube with a few drops of Tyrode-serum mixture and gently laid on the surface of an agar slant as described above so that about ⅓ of the surface is covered. Subsequent transfers are made at intervals of anywhere from the 6th to the 10th days by removing the tissue from a previously inoculated tube, mixing it with a fresh normal tunica, mincing them together, and allowing them to stand for 10 minutes before inoculating other slants. It is wise, at first, not to attempt to make more than three new slants from a single old one. Later, as growth picks up, five or six transplants can be made from a single seed tube.

During the last 8 months we have found that cultures started on tunica tissue can be carried over to young mouse embryo tissue and eventually to embryonic chick. In both cases the removal and preparation of the embryonic tissue is practised as for other methods of tissue culture.

Rapid examination for Rickettsia in such cultures is now made as a routine by the staining technique, first worked out in this laboratory by Dr. Attilio Macchiavello and not separately published. It is carried on as follows:

A 0.25 per cent solution of basic fuchsin is made either in a phosphate solution, buffered at 7.4 pH or in distilled water brought to 7.2 to 7.4 pH with sodium

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hydrate or sodium carbohydrate. Preparations are made by smearing a bit of tissue on the slide, drying gently by heat after drying in the air, and the fuchsin solution is filtered over the preparation through a coarse filter paper in a funnel. The fuchsin is left on the slide for 4 minutes, is washed off very rapidly with 0.5 per cent citric acid solution. The citric acid solution is poured on and off the slide and very rapidly washed with tap water. It is then stained for about 10 seconds with a 1 per cent aqueous solution of methylene blue. With a little practise and adjustment to individual laboratory materials this method gives excellent contrast stain, the Rickettsiae, intra- and extracellular, being stained red, the cellular elements blue. This method, though excellent for culture smears, is not successful in tissue sections of typhus animals for which the Giemsa method seems to be the only reliable technique at the present time.

RESULTS

By the method outlined we have started a considerable number of Rickettsia cultures from guinea pigs infected both with the murine and the classical types of virus. This has been done not only with the strains carried in passage for many years at The Harvard Medical School, but with strains isolated in Peking from patients infected with both the North China epidemic strain, which we consider identical with the classical European type, and the Manchurian strain of typhus which is identical with the murine or Mexican variety. These Chinese strains are being studied in more detail by Dr. Samuel Zia of the Peking Union Medical College.

The first transfer from infected animals to the agar cultures is usually easy to grow. Difficulties arise from the second to about the sixth subculture when growth may be relatively sparse and contaminations may be frequent, owing to the fact that workers just beginning with this method must develop a certain degree of dexterity in handling it. After the strains have once been carried through the fifth or sixth generations and the system of uniform production of the agar, control of the materials, and proper technique have been established in the laboratory, the matter becomes a routine procedure which a competent technician can carry on independently.

By this method both the murine and the classical European Rickettsiae grow equally well. But since the x-ray rat method is quite satisfactory for the production of large amounts of murine Rickettsiae we have concentrated, in the present study, largely on the classical European virus. It was necessary to determine whether tissues

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other than guinea pig tunica could be successfully used; whether prolonged cultivation on tissues other than the tunica, would maintain the virulence and specific pathogenic properties of the strain; and whether animals infected with living Rickettsiae from such agar cultures, after considerable periods of culture passage, were thereby rendered immune to subsequent inoculation with homologous passage virus. Finally it was important to find out whether killed Rickettsiae grown by this method would immunize to passage virus.

Incidentally, the cultures yield such large amounts of Rickettsiae of both strains that these can be used in suspension for serological experiments, such as for instance, the comparative agglutinative power of convalescent and immune sera for Rickettsia on the one hand, and proteus X bacilli on the other. These, and other immunological questions, are being investigated by one of us.

We have found in the course of over a year that mouse embryo is far more practical for Rickettsia cultivation on the agar slants, than is guinea pig tunica. The mouse embryos are taken at a stage when they have not exceeded the size of about 1 cm. in length, about one-third term. In other respects they are prepared as for making the tunica cultures, except that we have cut up the tissue in sterile test tubes of the shape and size of the ordinary 50 cm. centrifuge tube, instead of in Petri dishes, thereby considerably cutting down contaminations. Although, at first, chick embryo did not seem favorable and gave irregular and sparse results, later experiments carried out after the culture had been on agar tissue media for twelve or thirteen generations, resulted in excellent growth on the chick embryo. Since that time cultures have been carried in parallel, both on mouse embryo and on chick embryo. Very little immunological work has so far been done with the chick embryo cultures.

Inoculation of Animals with Agar Tissue Cultures

In order to determine the persistence of pathogenic properties, we have inoculated a large series of guinea pigs with Rickettsia cultures. All of these cultures were carried for 33 generations on Maitland flasks in the course of about 16 months and, subsequently, on agar tissue cultures for from 11 to 25 culture generations. In other words,

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none of these cultures had been less than 18 months on artificial media and some of them have been on artificial media as long as about two years.

Inoculation of guinea pigs with varying quantities of such cultures has always produced typical and severe temperature curves which do not differ from analogous curves produced by the inoculation of the original passage virus, except that the rise above 104° takes place very much more rapidly in the former, than in the latter cases, probably owing to the much larger numbers of Rickettsiae introduced with the culture material than with the blood and brain of infected ani-

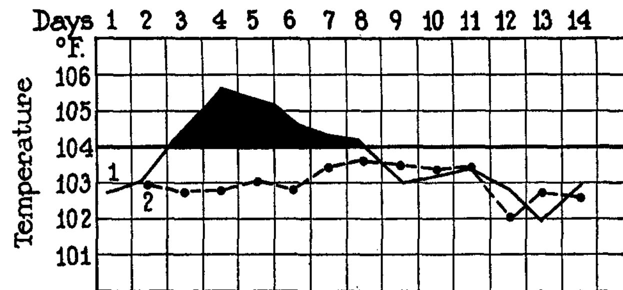

CHART 1

1. Temperature curve of guinea pig inoculated with 22nd generation agar culture (55th culture generation). ⅓ of one slant used.

2. Temperature curve of same guinea pig following immunity test with passage virus.

mals. Moreover, with the cultures many Rickettsiae are introduced in the free state ready to invade animal tissues, whereas, in the case of tissue inoculation, many organisms are intracellular and cannot infect until the cells in which they are contained have disintegrated. Charts 1 and 2 illustrate the types of reaction obtained in guinea pigs intraperitoneally inoculated with agar tissue Rickettsia cultures, the culture generation in each case being marked on the chart. There have now been carried out eighteen such experiments, all of them consistent in result. In eight the development of temperature was accompanied by transitory scrotal swelling. Control cultures were always taken from the inoculated culture on blood agar plates, and often on blood broth. In a number of infected guinea pigs, heart's

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blood cultures were taken on the 2nd or 3rd day after the rise of temperature and found sterile.

In one animal which had been inoculated with the contents of a tube which after 33 generations in Maitland had been carried for six generations on agar tissue culture, the left testicle was removed on the 4th day when the temperature had risen to 105.8°F. and Rickettsiae were found in the smears from the tunica. Heart's blood of this animal and emulsified tunica were injected respectively into two other animals. The animal receiving the heart's blood developed a mild but typical typhus reaction with a rise of temperature of 104.4°F. on the 6th day. The one receiving the tunica material of this animal developed a characteristic and severe typhus reaction. The animal inoculated with blood unfortunately

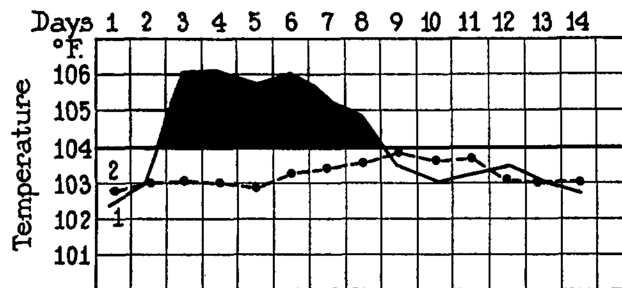

CHART 2

1. Temperature curve of guinea pig inoculated with 25th generation agar culture (58th culture generation). ½ of one slant used.

2. Temperature curve of same guinea pig following immunity test with passage virus.

died on the 6th day from pneumonia and could not be tested for immunity, but the one which had received the tunica and passed through a typical clinical course of guinea pig typhus was reinoculated with passage virus 23 days after it had been inoculated with the culture virus, suitable controls being inoculated at the same time. This animal was found immune, proving thereby that the culture disease had been a typhus infection, homologous with the passage virus.

In two animals, both inoculated with mouse embryo agar culture material of the eleventh agar generation, the brain was removed on the 11th day after typical febrile reactions. In both cases there had been slight scrotal swelling on the 4th day and, in both, the temperature had been maintained over 104°F. for 6 days and over 105°F. for 4 and 3 days respectively. Histological study of both brains showed numerous typical typhus brain lesions.

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In regard to the immunity conferred to passage virus by previous inoculation of culture virus, it was found that, of the eighteen experiments cited above, thirteen animals were completely immunized so that inoculation with passage virus about a month after inoculation with the culture virus resulted in absolutely no temperature reactions and no other signs of illness, during 21 to 29 days of observation. In four of the remaining five animals typical typhus developed after culture infection but on reinoculation with passage virus a month later they showed short periods of 1 to 3 days during which the temperatures touched 104°F. In no case could the reaction of the passage virus have been regarded as a typical infection but we mention these for the sake of completeness. These experiments were carried out during the very hot weather when many control guinea pigs showed slight fever. In the fifth case the original inoculation with the 24th agar generation was made with only a single loopful of the culture and the inoculated animal showed 3 days of temperature above 104°F., rising to 104.5° on the 7th day. Reinoculated 5 weeks later with a passage virus to which the controls reacted typically, this animal remained free of temperature throughout, except that on the 10th day its temperature rose to 104°F. We believe that even in this case the animal can be regarded as having been immunized by a culture infection quantitatively so slight as to result in only a very mild typhus. In this connection the immunological work going on at the present time is directed, among other things, to the determination of accurate dosage of Rickettsiae, a procedure rendered possible by the agar method.

Normal rabbits inoculated intraperitoneally with agar slant cultures acquired a positive Weil-Felix reaction in 7 to 10 days. Three of the animals, having a negative Weil-Felix before inoculation, reacted as follows:

1. Rabbit 3-86, on the 10th day following inoculation with a whole agar slant, gave a Weil-Felix of 1/20 ++++ 1/40 +++ 1/80 ++ 1/160 0.
2. Rabbit 4-06, also given a whole agar slant; Weil-Felix 10 days later: 1/20 ++++ 1/40 +++ 1/80 +++ 1/160 ++.
3. Rabbit, 4-12, given ⅛ of an agar slant culture; Weil-Felix 10 days later: 1/20 ++++ 1/40 ++++ 1/80 +++ 1/160 +++ 1/320 ++.

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Vaccination Experiments

The principle that animals can be actively immunized against typhus infection by vaccination with killed Rickettsiae has been conclusively established by a number of investigators. Weigl (8) has demonstrated the effectiveness of phenolized vaccines made of the intestinal contents of infected lice, and both Kligler and Aschner (4), as well as one of us with Macchiavello, have shown the same thing for formalinized Maitland cultures of Rickettsiae. The Weigl method, while unquestionably effective, does not lend itself, for reasons of technical difficulty, to the large scale vaccinations required for practical purposes in an epidemic disease. To a lesser degree, this criticism applies to the Maitland tissue culture methods. Although such cultures have been simplified and volumetrically enlarged by us, the yield of Rickettsiae is always limited by the fact that growth takes place only within and on the tissue particles, and there is no growth when more than a relatively small amount of tissue is employed. The x-ray rat method of Zinsser and Castaneda fulfills all the criteria of practical vaccine production, but unfortunately is not applicable to the classical European Rickettsiae.

It was for these reasons, chiefly, that we carried out the experiments which led to the method described in the preceding paragraphs.

The agar tissue culture method yields enormous quantities of Rickettsiae of either of the two known typhus strains. Moreover, the organisms so cultivated retain all the characteristics of passage virus. They produce typical infection in guinea pigs, which, after convalescence, are immunized to homologous passage virus. They give rise to typical brain lesions in infected guinea pigs and to Weil-Felix reactions in infected rabbits. The cultures retain these properties for at least two years without apparent antigenic dissociation. There is no theoretical reason, therefore, why they should not serve as "killed vaccine" as well as Rickettsiae obtained by other methods, with the added advantage of being easily available in any required amounts.

Vaccination experiments were therefore carried out. Some of these are shown in Chart 3.

In two further vaccinated animals of the "blood and brain" series

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of this experiment, the temperatures touched 104.2° on the 13th day, but promptly fell to 103.5° and 103.8° respectively on the 14th day.

It should be stated that in experiments such as those given above, the doses of passage virus used for reinoculation were always con-

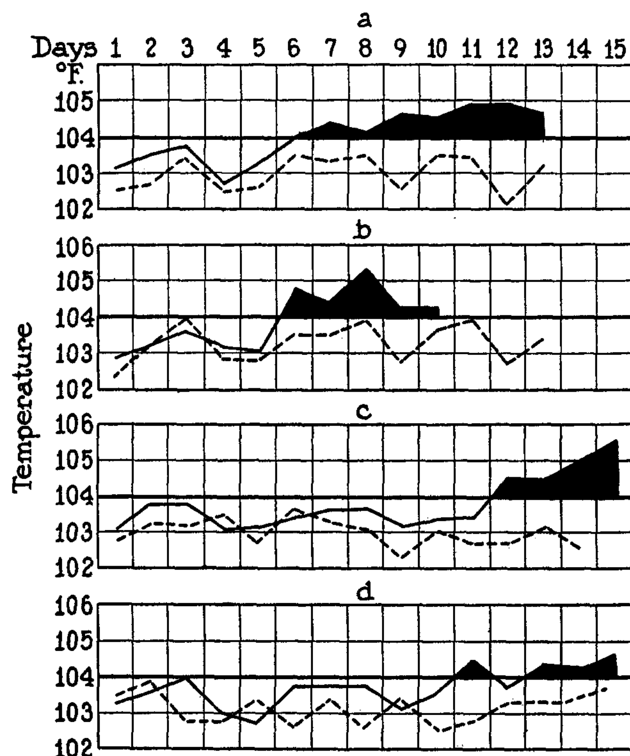

CHART 3. In curves *a* and *b*, the broken lines represent the temperature curves of two guinea pigs vaccinated with agar tissue culture Rickettsiae killed with 0.3 per cent formalin. They received subcutaneously three injections at 6 day intervals, the total amount administered representing about 1.3 agar culture slants. Reinoculation was carried out intraperitoneally with 1 cc. of a mixture of brain and defibrinated blood of European passage virus 6 weeks after the last vaccination. The unbroken lines are the curves of controls receiving the same amount of passage virus.

Curves *c* and *d* represent similar experiments, in which the reinoculation was carried out with smaller doses of passage virus, namely, 1 cc. of virulent defibrinated blood.

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siderably above the minimum infectious amount. Without a costly and individual titration in each case, it is impossible to state with exactitude the number of infectious doses administered. However, we have found in the past that blood plasma of typhus guinea pigs at the height of their temperature curves is infectious in doses varying between 0.05 and 0.1 cc., and since a considerable amount of virus has been found to adhere firmly to the red cells, it is plain that 1 cc. of defibrinated blood represents certainly not less, but probably more than ten infectious doses. When brain material is added to blood, the quantitative virulence cannot be estimated, but is distinctly higher than with blood alone.

It is clear from these experiments and others like them that guinea pigs treated with formalinized or phenol-killed Rickettsiae from agar tissue cultures are either completely immunized or rendered so much more resistant than normal animals that only a late and very slight reaction results from subsequent inoculation with large doses of passage virus. We believe that, in an infection as severe as typhus, degrees of immunization with killed organisms are, as in the case of the virus agents, almost directly dependent on the quantities of antigen administered. And by this method, unlike any of the others, the available amount of organisms is practically unlimited.

DISCUSSION

The importance of developing a safe prophylactic method against typhus fever requires no comment. The proposed methods, based upon the use of living virus (9, 10), are unsafe and unjustified until all efforts to develop killed vaccines have been shown ineffective. The principle that killed Rickettsiae can induce a considerable increase of resistance in animals has been demonstrated by a number of investigators, and some of those methods have been applied to man with results that are encouraging. It is obvious, however, that, as in the case of typhoid prophylaxis and other bacterial vaccines, a large accumulation of data is necessary before positive statements can be made.

Of the reports on typhus vaccination, the most encouraging are those of Rutten (11), who has employed the Weigl louse vaccines in the protection of missionaries of the Pères de Scheut, a Belgian mis-

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sion working out of Peking into central China. His figures, which we refrain from citing in detail, indicate beyond question that, while these vaccines have not completely suppressed the disease, they have, at any rate, much diminished the morbidity, have eliminated the mortality, and have rendered the cases which occurred, with but few exceptions, milder and of shorter duration. We do not claim, for the method described, any qualitative superiority over the Weigl or over the Maitland culture methods. We believe, however, that, quantity for quantity, the vaccines produced by the use of the agar tissue cultures are equal in immunizing power to the others, and that this method provides a simple and cheap technique for the production of any required amounts of antigen. Field studies only can determine its eventual usefulness.

Moreover, for those investigators who, like Blanc and Laigret, believe that the necessity for the rapid production of large amounts of vaccine in the face of epidemics justifies the use of living virus for human vaccination, our method furnishes a technique by which exact quantitative measurements of Rickettsiae can be substituted for the uncertain dosage of virulent animal organs. We are now studying the infectious and immunizing properties of exactly quantitated fractions of agar cultures and find that as little as one-eightieth of a slant will still produce typical febrile reactions in guinea pigs. On this basis at least eighty doses, probably many more, can be obtained from a single slant. The immunological data on this type of procedure will be reported at a later date.

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