CULTURING OF SOME PATHOGENIC RICKETTSIA ON ACELLULAR SEMISYNTHETIC NUTRITIVE MEDIUM

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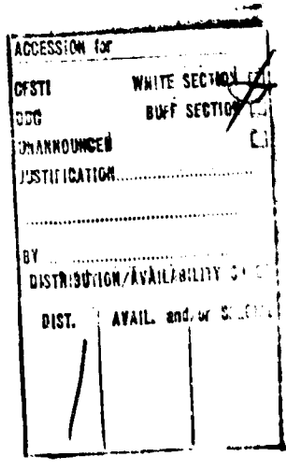

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CULTURING OF SOME PATHOGENIC RICKETTSIA ON ACELLULAR SEMISYNTHETIC NUTRITIVE MEDIUM

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In microbiology the opinion has long been held that all pathogenic human rickettsia, as well as filterable viruses, are biotropes, which can live and develop only in live or surviving cells and tissues. Hence, the dictum of O'Dyurua "Without life there is no life of virus" has become an axiom.

However, it is known that viruses and rickettsia by their nature are far from uniform: some approach bacteria in certain properties, while others are strictly intracellular parasites. It has been shown in our laboratories that the causative agents of the Rickettsia quintana and paroxysmal Rickettsia in the carrier organism can be detected both extra- as well as intracellularly. It is difficult to conceive of how enormous an accumulation of Rickettsia prowazeki in the intestines could be achieved only through intracellular development without the participation of cellular and hematic detritus. Therefore our attempts to cultivate the most plastic Rickettsia quintana on nutrient media of complex composition were wholly logical.

At the first stage of formulating such a nutrient medium through numerous observations all the substances were first found and then selected which promoted the most extended survival of Rickettsia. Investigation showed that it is useful to formulate a medium of three components: human blood, chicken yolk and milk (the KZhM /kurinyy zheltok moloko/ medium).

By changing the doses of components in the medium, its pH, the conditions of aeration, etc., we ultimately succeeded not only in achieving prolonged survival of Rickettsia, but also their multiplication (Petrova, 1957; Pshenichnov, 1957, 1959; Pshenichnov and Plaksina, 1963). In this way the problem of the possibility of growing human pathogenic Rickettsia on nutrient media of complex composition was fundamentally solved.

However, initially we could not get stable results and culture growth was observed only in 5.7 per cent of the seedings.

The results of chromatographic study of medium composition pointed to the instability of the formulation of its components, the absence in definite amounts of such amino acids as glycine, norleucine, oxyproline, hydroxyproline, and the presence in the medium of labile fractions, some of which had become decomposed at the time of Rickettsia multiplication. From this point we began to add the following enriching agents to the medium: 1) 1-5 per cent yeast dialysate or "manifestor", the filtrate of a 25-day culture of Bac. mesentericus vulgaris; 2) a complex of vitamins A, B_1, B_{12}, and C in the form of an aqueous solution on the basis of 0.001 mg of vitamin A, 0.2 mg of vitamin B_1 and C, and 1.5 micrograms of vitamin B_{12} per 4 ml of nutrient medium; the vitamins were added on the 7th and 14th day of culturing; 3) 10-20 per cent synthetic medium No 199; 4) a ten per cent aqueous extract of tissues of susceptible animals (guinea pig, white mouse) and body lice; 5) 5 per cent amniotic fluid of bovine embryo.

To inactivate the native inhibitors preliminary freezing and thawing of the medium was carried out and hemolyzed blood was used.

Many observations (more than 3000 seedings) showed that yeast dialysate, "manifestor" and aqueous extract of insect tissues had no favorable effect on Rickettsia growth.

Amniotic fluid of bovine embryo retarded virus growth. Addition of vitamins and extract of guinea pig organs promoted Rickettsia adaptation to some extent. But especially favorable results were achieved by the addition of 10-20 per cent medium No 199. Not only was the content of amino acids, salts, and growth stimulators already existing in the medium increased, but there was also added such amino acids as norleucine, oxyproline, hydroxyproline which in definite amounts in the previous versions of the medium as a rule were not present. In the presence of medium No 199 the Rickettsia culture was held quite constant and virus accumulation was more abundant.

It is possible through further experiments to establish that pH 7.2-7.25 is the most favorable for Rickettsia growth.

Considerable improvement was seen in the results of Rickettsia culturing in which the method of rotating test tubes was used. We placed culture-containing test tubes in the DAG-1 apparatus, in which the rotation was 12 revolutions per hour. Apparently, the growing Rickettsia colony suddenly released into the surrounding environment products of metabolism and autolysis, and thus established around it a zone obstructing processes of assimilation. Rotation of the test tubes led to a constant mixing of the medium and in this way assured the steady access of fresh nutrient substances required for normal life activity of Rickettsia.

Further observations established that the causative agents of trench fever are facultative anaerobes.

This modification of the medium (containing 10-20% of medium No. 199, and sometimes a complex of vitamins and extract of guinea pig tissue), and also its constant mixing in test tubes and culturing with access to air contained in the test tube assured wholly satisfactory results. Thus, in the medium of initial composition Rickettsia growth was obtained only in 5.7% the seedings, while growth was noted in 60% of the cases using the medium modification described, and in some experiments -- in 90%.

A definite gradation in virus development on artificial nutrient medium was established. After small Rickettsia doses were placed in the test tubes colored preparations in the medium were detected in by no means each field of view. During the first week they appeared to disappear entirely. In all probability their adaptation to the medium took place. Then the phase of logarithmic growth set in, and by the second to third week distinctive microcolonies appeared in the medium, consisting of clusters of large or small numbers of Rickettsia (Figure 1). During this period their number mounted to 250,000,000 - 400,000,000 per ml of medium.

Subsequently, the virus concentration was reduced and by the 30th - 40th day viable Rickettsia could not be detected in the cultures. Adaptation of Rickettsia promotes their better growth and accumulation in the medium.

It must be noted that the causative agents of trench fever were especially successfully cultivated in the medium during the spring-summer; during autumn and winter when cultured in a medium prepared exactingly to the same formulation, unstable results were obtained. This is best accounted for by the effect of the season of the year on the quali-

tative content of amino acids, trace elements, vitamins, and other growth factors in the original ingredients of the medium (Grebennikov et al., 1963; Kugenev and Medvedeva, 1962; Lebedeva, 1963). However, although it was possible carefully to culture Rickettsia during the spring-summer period, adapted strains can be maintained in the medium all year long.

Strains of trench fever Rickettsia adapted to the liquid nutrient medium were grown on a variant of the medium thickened with agar. Rickettsia colonies were small, up to 1-1.5 mm in diameter, gray, with a slightly coarse surface (Figure 2). The colonies appeared to interlock in the medium and with difficulty were separated from its surface.

In addition to trench fever Rickettsia experiments on cultivation in the modified medium were carried out with the causative agent of paroxysmal Rickettsia (the Marta strain). It was found that this species of Rickettsia can also be cultivated on an artificial medium.

This data allowed us to assert that reproduction of these pathogenic Rickettsia is possible on synthetic nutrient medium, apart from living cells. This was confirmed by the following facts. First of all, when the preparations from the nutrient medium were examined under a microscope, the cells on which virus development was to have been expected, were extremely rare and unquestionably could not have provided for abundant accumulation of Rickettsia. Secondly, during the period of maximal Rickettsia growth, that is, by the first-second week the shaped elements of the medium were converted into detritus. Thirdly, the growth of Rickettsia was obtained also on variants of the medium, in which the cells had been previously degraded through repeated freezing and thawing or removed by centrifuging. Based on these observations it can apparently by suggested that causative agents of trench fever, and also paroxysmal Rickettsia are facultative biotropes since their biosynthesis is achieved not only in the live organism, but also in conditions of extracellular nutrient medium.

The method of culturing on a nutrient medium not only improves prospects for preparing different biopreparations, but it will also promote the study of Rickettsia physiology. Thus, even in the first of our observations it was noted that in the development of Rickettsia the amino acid composition of the medium changes: in it the content of such free amino acids as histidine, lysine ,

Figure 1. Rickettsia quintana in liquid nutrient medium on 21st day of growth. Staining after Romanovskiy-Gimze. X 1400.

Figure 2. Colonies of Rickettsia quintana on a solid nutrient medium, 7th day of growth. Staining according to Romanovskiy-Gimze. X 35.

Figure 3. Rickettsia prowazeki (strain E) in liquid nutrient medium, 21st day of growth. Staining according to Romanovskiy-Gimze. X 1400.

alanine, arginine, tyrosine, and isoleucine is reduced; at the same time in several cases, a new amino acid was found to appear in the cultures -- tryptophan. Experiments in this area are continuing.

The method we suggested for studying metabolism has appreciable advantages over the method of Bovarnick and Schneider (1960), who studied metabolism in Rickettsia under conditions of short-term survival, and not during the process of development. Having obtained successful results in raising two species of Rickettsia, we proceeded to experiments on culturing the causative agent of typhus, which is usually localized intracellularly in the organism of the carrier. From a large number of experiments, we performed it was possible to record survival and some accumulation of Rickettsia prowazeki in synthetic medium only in individual cases. For instance, in three successive passages on the original variant of the medium we (together with Moskova) succeeded in detecting Rickettsia in small amount by the 14th-28th day following inoculation, although we did not succeed in reinforcing the virus culture in subsequent passages.

In recent years Bovarnick and Schneider (1960), Bovarnik and Miller (1950), Zubok (1960) et al, have shown that Rickettsia prowazeki and muzer exhibit distinctive enzymatic systems. In particular, they form serine from glycine and formaldehyde, incorporate a definite amount of C^{14}-labeled amino acids, etc., that is, capable of a degree of independent synthesis.

We carried out the last experiment with a modified variant of the medium. Typhus vaccine drain E was used for the inoculation, as the most plastic and readily growing drain in chick embryos. Into the medium was placed 0.1 ml of chick embryo yolk sac with abundant virus content, which upon passage was receded to the extent of 0.2-0.5 ml of a 25-day Rickettsia culture in 4-5 ml of fresh nutrient medium. The control over development of virus in the medium was carried out by the following methods: by microscopic examination of stained preparations from the medium on the 7th, 14th, and 21st day following inoculation, symptoms of the virus in body lice by the epidermomembrane method, by immunological shift in the sera of guinea pigs on the 21st day following intraparenteral administration of passage material, and by the oveculture.

In our experiments, we succeeded in sustaining Rickettsia prowazeki for about a year on the nutrient medium. During this time the eleven passages were performed. Evidently, this period is not elimiting one (culturing was discontinued for technical reasons). During the entire experiment Rickettsia was detected by microscope (from individual organisms to clusters) in the nutrient medium, and so sensitive a biological reagent as body louse was detected; immunological shifts were brought about in guinea pigs (up to 1:8 - 1: 128) and by the fifth passage the specific death of chick embryos was induced.

The virus content in the medium in different passages was dissimilar: during the first passages it was high, then the Rickettsia concentration fell off. At the end of the experiment, that is, in 7-8 months, the amount of Rickettsia in the medium began to increase again: the percentage of smears containing virus rose 24-30%, 2-5 % of the insects were infected upon single feeding through membrane, administration of the material led to the appearance in the guinea pig serum of complement-fixing antibodies to Rickettsia prowazeki in a titer up to 1:32 and only for unknown reasons was it impossible to detect the virus in chick embryos.

Two suggestions are possible: either mechanical transfer of Rickettsia from one test tube to another took place upon passage, or we actually observed adaptation and development of the virus in new habitats unfamiliar to it.

Upholding the first assumption is the fact that the original seeding material underwent dilution at least to 25 million-fold. In addition, it is known that typhus Rickettsia in general are poorly stable and cannot be preserved under thermostat conditions. The dynamics of virus behavior in a medium, the increase in Rickettsia concentration upon passage, and formation of microcolonies (Figure 3) testified to its gradual adaptation and development.

Therefore, it can be assumed that typhus Rickettsia are also not absolute biotropes, that they can adapt to some extent to conditions of life apart from live or surviving cells, if a medium containing all the complex array of nutrients needed for their life activity is chosen. It is possible that further advances in biochemistry will permit the synthesis and provide the laboratory with such nutrient media in which biosynthesis and reproduction of pathogenic Rickettsia will be easily achieved even without live or surviving cells.

Conclusions

1. It has been established that trench fever Rickettsia and paroxysmal Rickettsia can be successfully cultivated in liquid and in solid variants of a complex polysynthetic noncellular nutrient medium.

2. It was shown for the first time that strain E of typhus Rickettsia not only survived for an extended time, but adapted to some extent and multiplied in a synthetic noncellular nutrient medium.

3. This data refuted the opinion that all pathogenic Rickettsia are strict biotropes, the life and development of which proceed only in living and surviving cells, and unfolds the prospects for improvement in methods of culturing Rickettsia and in preparing the corresponding biopreparations.

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