CULTIVATION OF EATON'S AGENT (Mycoplasma pneumoniae) ON ARTIFICIAL NUTRIENT MEDIA

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The causative agents of the respiratory diseases of man, animals and birds belong to an extensive group of microorganisms within the family Mycoplasmataceae. The etiological role of microplasms in the various respiratory diseases of animals and birds was known long ago, but the etiological significance of the causative agents of this family in human disease became known only recently.

In 1944 Eaton [3] isolated a filterable agent (called Eaton's agent) by transferring mucus from animals ill with pneumonia to cotton rats. Because of the variable results obtained in transferring this agent to cotton rats and hamsters, it was very difficult to keep the agent under laboratory conditions, and owing to the absence of cytopathogenic effects in tissue and embryonic cultures, there were no criteria by which to determine the transferability of the causative agent until the immunofluorescent method was applied for determining the serodiagnostics of the agent [5]. Until 1962 Eaton's agent was thought to be related to the filterable viruses, but in 1962 it was shown to be a member of the family Mycoplasmataceae [1]. For the first time a culture of the agent was obtained growing on artificial cell-free medium in a form characteristic of PPLO (microplasm) colonies and capable of being grown under these conditions in subcultures. The agent was called Mycoplasma pneumoniae.

The etiological role of M. pneumoniae in primary, atypical, pneumoniase was demonstrated in a whole series of virological, serological and, in later years, microbiological investigations and also by the results obtained from experimentally inoculated volunteers [2]. By employing such diverse research methods the etiological role of Eaton's agent was also demonstrated epidemiologically.

The characteristic features of M. pneumoniae are the following: growth in the form of colonies typical of PPLO on artificial nutrient media adequate for the nutritive requirements of microplasm (PPLO); the presence of a microstructure, specific to microplasm, in the composition of the colonies; the capacity of being transplanted in subcultures to produce hemolysis of the red blood cells of man and guinea pigs; and an antigenic property—in contrast to certain other forms of microplasm of human origin (Mycoplasma hominis and II, Mycoplasma fermentans, and Mycoplasma salivarium) A standard medium is recommended [1] for cultivating M. pneumoniae; it contains Difco brand PPLO agar (70 ml), 25% yeast hydrolyzate from dried Fleischman's type 20-40 yeast (10 ml), normal horse serum (20 ml), penicillin (100,000 Dosage Units/ml, gallium acetate (final dilution 1:2000), and amphotericin (0.5 mg/ml) for preventing contamination by harmful bacteria.

Though the subject has been intensively studied abroad (USA, England, Holland, etc.), no research on the biology of M. pneunoniae and the part it plays in the etiology of respiratory diseases has been carried out in the USSR. A study of the role of M. pneumoniae in fatal respiratory infections is of considerable interest for epidemiology, clinical work and the therapy of these infections.

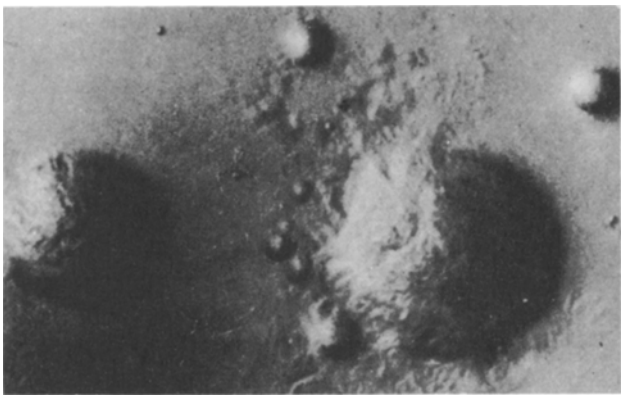

Fig. 1. Colonies of M. pneumoniae on semisolid nutrient medium. Phase contrast microscopy, magnification 90 X.

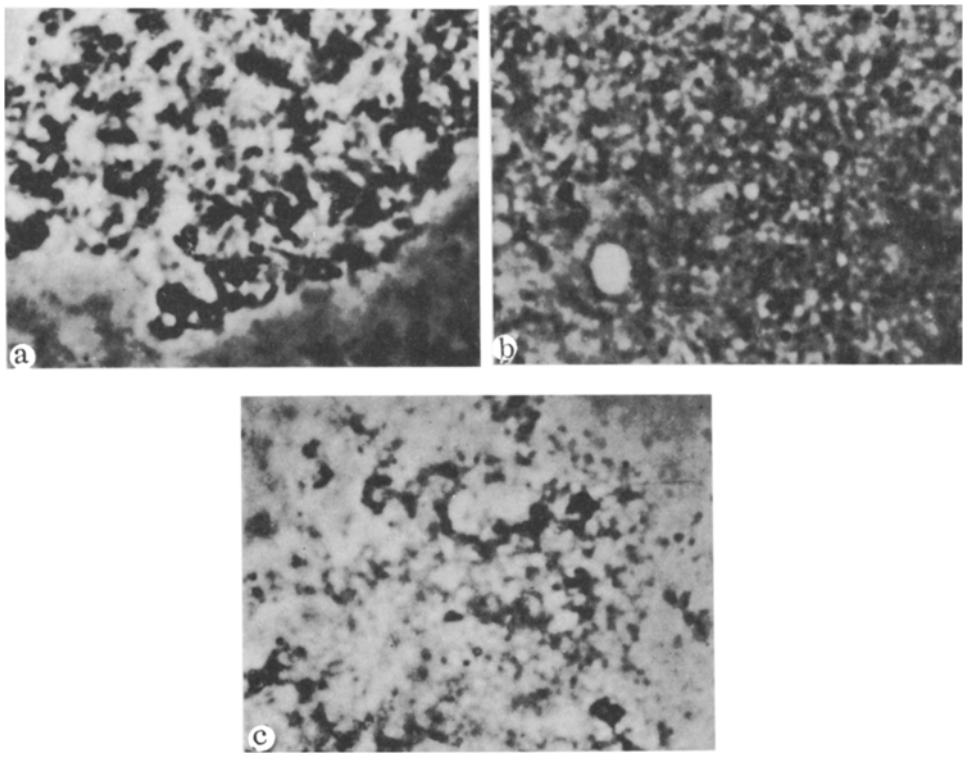

Fig. 2. Microstructure of M. pneumoniae on semisolid nutrient medium. a) Conglomeration of granular cultures, shapeless bodies, and isolated vacuoles; b) granular structure and vacuoles of various sizes; c) granular structure and small vacuoles, adjoining microcolonies of vesicular form. Phase contrast microscopy, magnification 1350 X.

The aim of the present work was to study the biology of the standard strain FH of M. pneumoniae.* While undertaking this work we explained the possibility of adapting the causative agent to empirical nutrient media and searched for the most effective method of culturing the agent.

*The strain was kindly given to us by Professor R. Chénok, to whom we express our sincere thanks.

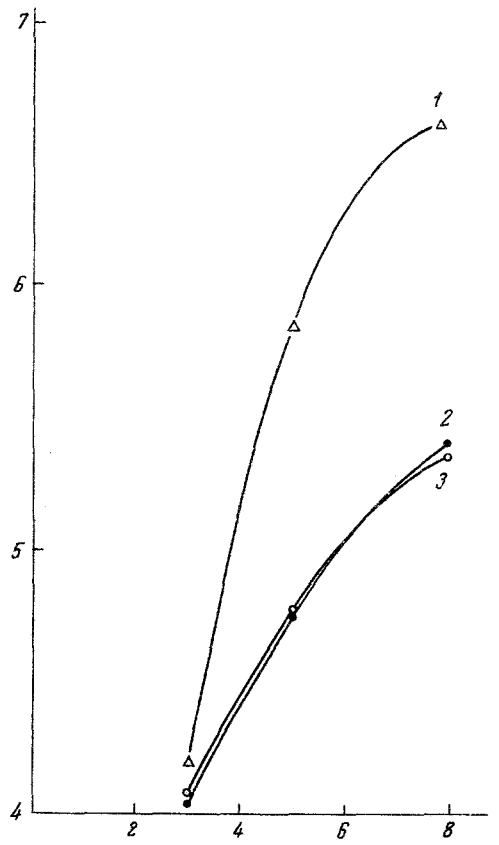

Fig. 3. Dynamics of the increase in viable particles of M. pneumoniae depending on the conditions of culturing. Ordinate) logarithm of number of viable particles in 1 ml medium; abscissa) time of culturing in days. 1) Cultivation in two-phase nutrient medium; 2) cultivation in liquid nutrient medium; 3) cultivation in liquid nutrient medium containing dextrose.

EXPERIMENTAL

The strain FH was grown on a standard Difco brand PPLO and, in parallel tests, on the empirical medium which has been successfully used for culturing the L-forms of various species of bacteria. Investigations were made on the following media: liquid, semiliquid (0.3%) and semisolid (1.3%) agar prepared by tryptic digestion of ox heart muscle with the addition of freshly prepared yeast hydrolyzate, 20% normal horse serum, 500 D.U./ml penicillin and gallium acetate (to a dilution of 1:2000). Transplanting was carried out by transferring blocks of agar bearing colonies onto the surface of fresh nutrient media [4] or by implantation into liquid or semiliquid cultures. Cultivation was carried out under aerobic conditions at 37°C for 6-7 days. The growth of the colonies was determined through a low power microscope. The microstructure of the individual colonies was examined by phase contrast microscopy at a magnification of 1350×.

RESULTS

The colonies growing on the standard PPLO Difco medium (Fig. 1) showed very little difference in morphology and size from those of M. pneumoniae grown on empirical solid medium. The colonies were round and erupted on the surface of the agar. Their size depended on the intensity of the seeding; single, isolated colonies were sometimes large, and in places where they had been deeply sown the colonies grew in groups. The surfaces of the colonies were somewhat granular.

On semiliquid and liquid media the growth of colonies visible to the naked eye was not recorded. The colonies on semisolid media consisted of small granules, small spherical bodies and shapeless masses with indefinite structure; they often contained a large number of vacuoles of various sizes and shapes which imparted a vesicular appearance to the structure (Fig. 2 a-c).

The microstructural elements of Eaton's agent growing on semiliquid medium did not differ fundamentally from those on the agent growing on semisolid medium. Microcolonies identical to those described above, but considerably smaller in size, were often encountered. Microstructures in the form of granular masses, granules, small and comparatively large spherical bodies were found on the liquid medium, but very small or large vacuoles were rarely seen. On the solid medium the agent was kept at 4° for ten days after the colonies appeared.

A definite capacity for adaptation to the semisolid medium described above was recorded in the FH strain examined. Initially, this strain gave an abundant growth of small colonies on solid, standard medium consisting of PPLO agar and Difco yeast extract. On the semisolid medium only isolated colonies developed, which were larger in size and retarded in growth when compared with the colonies grown on standard medium for 2-3 days. As a result of a gradual adaptation and selection, the FH strain began to grow well on an empirical medium. It was transferred to the given medium (24 transfers) in the course of six months, and it produced an abundant growth of comparatively large colonies in 3-4 days.

If liquid nutrient medium was poured over the surface of the agar in which the colonies were growing, the number and size of the colonies sharply increased, and their viability under these conditions at 37° was maintained for more than two weeks from the time of sowing.

In a parallel study of the growth on media containing various sera (bovine, guinea pig, rabbit and polyvinylpyrrolidone, an artificial substitute for serum) growth was observed only on the medium containing rabbit serum. In this instance the colonies were no different from those growing on media containing normal horse serum, but they transferred better.

In order to obtain a biomass of M. pneumoniae, needed for immunization and for obtaining antigens, we carried out comparative culturing on three media: 1) liquid medium (15 ml) prepared in test tubes according to the above-described prescription; 2) a similar medium with the addition of 1% solution of dextrose; 3) a two-phase medium consisting of two layers in flasks. The first layer was a semisolid agar (15 ml) onto which the FH culture in 0.5 ml of liquid medium was poured. After three days' growth, the second layer of liquid medium was poured over the surface of the first layer and the growth allowed to continue for 8-10 days with shaking twice daily. The intensity of the growth on the medium used was estimated by means of hemolytic tests.

Because of the capacity of M. pneumoniae to bring about hemolysis of the erythrocytes of man and guinea pigs, a hemolytic test was set up, the nature of which consisted of a 3-4 days' growth of the colonies on the solid medium with the subsequent addition of a layer of 4-5% blood agar [6]. After 24-48 h a zone of β-hemolysis, easily visible to the naked eye, appeared around the colonies of M. pneumoniae.

An estimate, made with the assistance of the hemolytic test, of the number of viable microorganisms produced (Fig. 3) led to the following conclusions. For all three media examined, the growth curve rose gradually to the 8th day of cultivation. The intensity of growth on the liquid medium and on the same medium with the addition of dextrose were approximately the same. There was a considerably greater intensity of growth on the two-phase medium.

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