

LONG-TERM CULTIVATION OF *N. GONORRHOEAE* IN TISSUE CULTURE

BY

M. GAVRILESCU, M. LAZĂR, I. POROJAN, AND T. CÎRCIUMĂRESCU

Centrul Dermato-Venerologic, București 10, Romania

The possibility of cultivating *N. gonorrhoeae* on artificial media aids its isolation for diagnostic purposes and microbiological study. Numerous growing media and maintenance or transport media, have been described, each trying to meet the nutritive requirements of *N. gonorrhoeae*, which is a very fragile organism outside the human host.

During recent years tissue cultures have been used as culture media for a great variety of viruses and bacteria. The gonococcus has been grown in tissue cultures in order to study its phagocytosis *in vitro* or its sensitivity to antibiotics following phagocytosis (Thayer, Perry, Field, and Garson, 1957; Meyer-Rohn and Rohde, 1961; Rohde and Meyer-Rohn, 1961).

In the present paper we report a study of the behaviour in time of gonococci grown in tissue cultures and the maintenance of some strains by successive subcultures.

Materials and Methods

In our experiments KB cell cultures were prepared according to the trypsinization procedure of Dulbecco and Vogt (1954). The medium from the storage culture flasks was removed and replaced by a 0.12 per cent. trypsin solution in PBS (phosphate-buffer solution); the flasks were incubated for 10 min. at 37°C., and the liquid centrifuged for 10 min. at 1,000 r.p.m.; the cellular sediment was washed with PBS and centrifuged again to remove traces of antibiotic. The sediment was suspended in the growth medium which contains — according to Aderca and Ianconescu (1962) — the following: Hanks's solution (Hanks, 1955) and the solution of Earle, Schilling, and others (1943), 35 per cent. each; 2.5 per cent. lactalbumin hydrolysate solution, 20 per cent.; calf serum, 10 per cent.; M/5 glutamine solution, 1 per cent.; yeastolate 0.10 per cent. Antibiotics were excluded from the medium. A concentration of 100,000

cells per ml. medium was obtained and 1 ml. amounts distributed to each culture tube. These were incubated at 37°C. in a stationary sloping position. The cells settled and grew on the inside lower wall of the tube forming a continuous cellular monolayer. Ordinarily the tubes can be used after an incubation of at least 48 hrs, an interval which is long enough to permit the elimination of possibly infected tubes. The tubes may be used for a maximum of 5 to 6 days after preparation.

The cultures of *N. gonorrhoeae* were obtained on solid Peizer-Steffen medium from cases of gonorrhoea in men and women. After the customary 48 hrs incubation on this medium the colonies were transferred to the cell culture tubes which were then re-incubated. For each strain of gonococcus at least five tubes were inoculated.

Results

After 24 hours the macroscopical examination of the tubes shows in the liquid medium rather large and numerous pellicles, which being stirred break up in smaller particles. From the third day on, these pellicles gradually become more friable so that eventually the medium turns uniformly muddy.

The cell culture is also affected concomitantly with the above changes. Within the first 24 hours the cellular layer presents here and there empty spaces caused by the detachment of cells from the walls, a phenomenon which becomes intensified on the second day and is completed on about the fifth day, when no more cells adhere to the glass wall.

Smears made from the liquid media were fixed by means of methanol and stained with Gram stain, methylene blue, or Giemsa stain. Examination after 24 hours reveals massive clumps of gonococci, the microscopical expression of the pellicles which were visible to the naked eye. These neisserian clumps seem to be disposed upon a material substratum composed of detached cells or of their remains. But often this substratum can no longer be seen, being literally covered by the organisms. After 48 hours these gradually diminish in the smears and there is

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an increasing production of amorphous material, somewhat granular or reticular, and staining weakly (Figs 1 and 2).

About 6 to 7 days after the inoculation the amorphous substance predominates in the smears, while isolated gonococci still persist here and there. The disappearance of gonococci does not occur at

a definite time, but is mostly complete after 2 weeks. Sometimes the organisms persist in culture for a long time; in one case they were still present after 88 days.

It is worth mentioning that subcultures are always possible from these gonococcus cultures. At present we have maintained 3 such strains, the oldest for 175 days since the first inoculation. For technical reasons

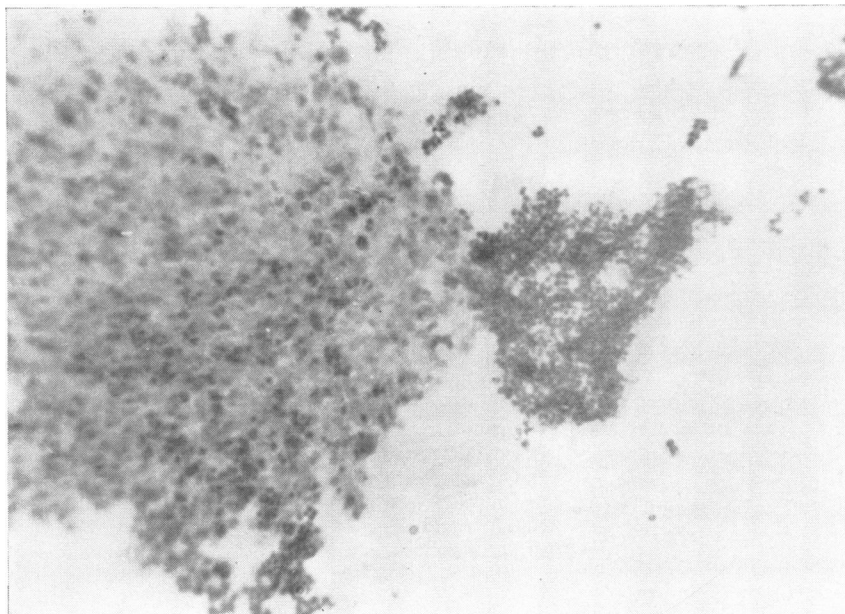


Fig. 1—*N. gonorrhoeae* in tissue cultures; clumps (right) and isolated colonies on amorphous substratum (left). Giemsa stain.

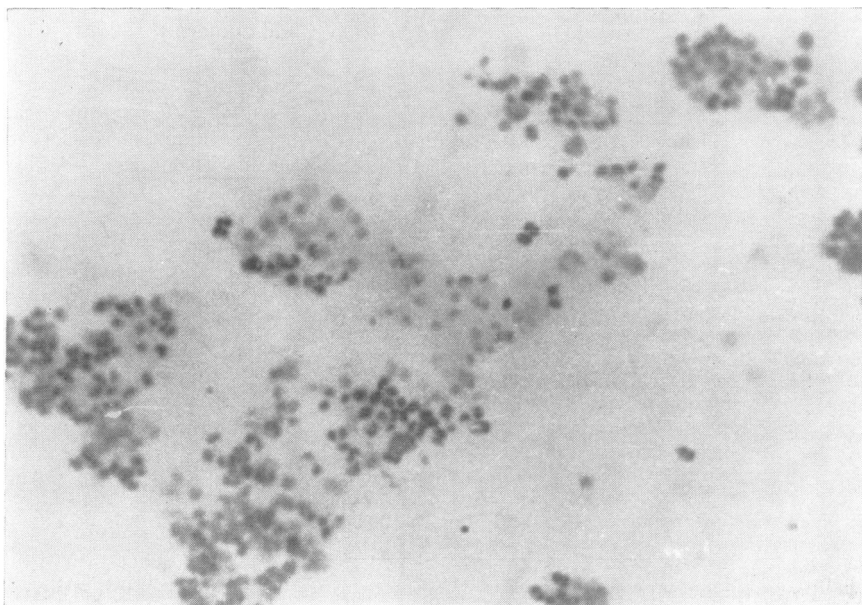


Fig. 2—Colonies of *N. gonorrhoeae* in tissue cultures; some organisms have lost their staining capacity. Giemsa stain.

subcultures were made every 7 days, but experience has shown that there is no optimum time for this. Subcultures can be grown at long intervals after inoculation. Positive transfers have been made after 23, 26, 31, 34, 58, 65, 74 and 88 days, even from tubes which showed no growth in stained smears. The significance of this fact will be discussed later.

Discussion

Our data show that the gonococcus grows and keeps in good condition in tissue cultures. We must, however, specify that this development depends on two factors: the living cellular substratum and the growth medium. In our experiments we generally used KB cells, but the cultures obtained on other cell types (HeLa, rat embryo), were as good. It is very important to have a living cell culture; attempts to cultivate gonococci in tissue cultures which had previously been killed by heating at 45°C. for 1 hour, gave scanty positive results.

The cell-free liquid medium failed to support the growth of gonococci. It is also obvious that the gonococcus is directly influenced by the composition of the medium. In our medium, which is entirely different from that used by Thayer, Perry, Magnuson, and Garson (1957) and by Meyer-Rohn and Rohde (1961), the organisms developed in all tubes in the best possible conditions. In a supplementary trial we inoculated in parallel tissue cultures prepared with other growth media, *i.e.* TC Medium 199 (Difco) or TC Medium Scherer (Difco), both with an addition of 20 per cent. calf serum. This experiment showed that in tissue cultures with the former medium the gonococcus grew as well but more slowly, but in the latter not at all. It should be mentioned that multiple bacteriological screenings performed at various stages during the investigation, enabled us to rule out any suspicion of contamination with common bacteria.

The cultivation of gonococci in tissue cultures is interesting for the following reasons:

- (1) An extremely abundant growth of organisms is obtained 24 hours after the inoculation which enables antibiotic sensitivity tests to be prepared one day earlier than by routine methods.
- (2) The method permits strains of gonococci to be maintained for long periods, thus facilitating various biological, immunological, and chemosensitivity studies.

Our results show that gonococci grown in tissue cultures gradually undergo an involution. The appearance in cultures of the amorphous, slightly granular substance as the numbers of organisms

decrease suggests that they may have undergone an "L-form" change. This hypothesis is supported by the ability to get positive sub-cultures, as well as by the results of sub-culture on Peizer-Steffen solid medium on which typical gonococcal colonies appear. These preliminary suppositions are to be elucidated by future investigations.

Summary

N. gonorrhoeae cultures on Peizer-Steffen solid medium have been transferred to KB cell cultures in the presence of an antibiotic-free nutrient medium. After 24 hours incubation at 36°C., a rich culture of gonococci is obtained. Subcultures are made every 7 days. Gonococci persist for a long time and subcultures can be obtained as long as 88 days after the first inoculation. By means of successive subcultures, three gonococcus strains were maintained for several months, the oldest for more than 5 months after isolation. The essential growth factors are represented both by the living cellular substratum and by the nutrient medium. Gonococci do not grow on the nutrient medium alone, and growth is very inconstant and poor on tissue cultures killed by heat.

The cultivation of *N. gonorrhoeae* in tissue cultures is of practical value because of the possibility of reducing by one day the time for carrying out antibiotic sensitivity tests, and of facilitating the long-term maintenance of strains.

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Maintien à long terme des souches de gonocoques aux cultures tissulaires

RÉSUMÉ

A partir des cultures de gonocoques sur le milieu solide de Peizer-Steffen on fait le transfert de l'organisme aux cultures cellulaires de type KB, en présence d'un milieu

nutritif exempt d'antibiotiques. Après une incubation de 24 heures à 36°C. on obtient de très abondantes cultures de gonocoques. Habituellement les passages ont été pratiqués chaque semaine mais on a pu répéter cette manœuvre avec succès même jusqu'à 88 jours après la primo-inoculation. Par de tels passages on maintient 3 souches de gonocoques, dont la plus ancienne dépasse 5 mois depuis l'isolation du germe.

Les facteurs indispensables pour la croissance semblent être les cellules vivantes et le milieu nutritif. Sur le

seul milieu nutritif le gonocoque ne croît pas; de même, les cultures cellulaires qui ont été tuées par la chaleur donnent de très faibles et inconstants résultats.

Les auteurs considèrent la méthode de la cultivation du gonocoque aux cultures tissulaires d'un grand intérêt, en premier lieu pour la réduction d'un jour du temps destiné à l'antibiogramme. Secondairement, cette méthode assure le maintien à long terme des souches de gonocoques, isolées au laboratoire, permettant ainsi les études biologiques les plus divers de l'organisme.