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COLLEGE OF VETERINARY MEDICINE
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TRANSLATION NO. 6

Translated from Russian by Virginia Ivens

Karapetyan, A. E.
1960. A method of cultivation of Lamblia[1]. Cytology 2:379-384.

Transliteration:
Metodika kul'tivirovaniya lambliĭ[1]. Tsitologiya 2:379-384.

In 1859 a Kharkovian scientist, D. F. Lambl, discovered protozoa for the first time in the excrements of patients suffering with diarrhea. They were later named Lamblia intestinalis after the discoverer. Several investigators (Schill, 1926; Chatterjee, 1927; Lippi and Mastrandea, 1958) have failed in their attempts to culture Lamblia in artificial nutrient media. We (Karapetyan, 1957) also failed to culture Lamblia in developing chicken embryos.

Parasitological examinations in the clinic revealed that Lamblia was found, as a rule, in association with other intestinal parasites and microorganisms. We examined samples of duodenal contents from 22 patients suffering with lambliosis. In 20 samples we found yeast-like fungi with the Lamblia and obtained from them 16 isolations belonging to the genus Candida. We identified 6 specifically: 3 isolations were Candida guilliermondi; 2, Candida albicans; 1, Candida tropicalis. We also obtained 4 isolations of the genus Torulopsis.

Brygoo (1952), Zil'berman (1956) and Cheboksarova (1958) also reported finding yeast-like fungi in duodenal contents. On the basis of these data, we believe there is a synergistic relationship between Lamblia and the yeast-like fungi in the intestine. If this is true in vivo, then it is only natural to make use of the fungi in attempting to culture Lamblia in vitro. With this in mind, attempts were made in 1959 to culture Lamblia with the fungi.

From 22 specimens of duodenal contents, we succeeded in isolating and maintaining 3 cultures of Lamblia with Candida guilliermondi. In 7 months, more than 20 passages were made, and at the present time the cultures are being maintained in the laboratory. A method of culturing Lamblia follows.

NUTRIENT MEDIA

The media contain salt solutions, chicken embryo extract, human serum, Hottinger's digest, and antibiotics.

Two salt solutions were used: Hanks' (NaCl - 8.0 g, KCl - 0.4 g, $MgSO_4 \cdot 7H_2O$ - 0.2 g, $CaCl_2$ - 0.14 g, KH_2PO_4 - 0.06 g, $Na_2HPO_4 \cdot 2H_2O$ - 0.06 g, $NaHCO_3$ - 0.35 g, glucose - 1.0 g, phenol red 0.5% - 4 ml, and 1000 ml of double distilled water) and Earle's (NaCl - 6.8 g, KCl - 0.4 g, $CaCl_2$ - 0.2 g, $MgSO_4$ - 0.1 g, NaH_2PO_4 - 0.125 g, $NaHCO_3$ - 2.2 g, glucose - 1.0 g, phenol red 0.5% - 4 ml, and 1000 ml of double distilled water).

[1]Reported November 25, 1959, at the Leningrad Parasitological Society Conference.

The chicken embryonic extract was prepared in Hanks' or Earle's salt solution by the method established for culturing tissues (Melnick, 1956; Levi, 1957). Human serum was inactivated by heat at 56° for 30 minutes. The serum and salt solutions were sterilized by being filtered through a Seitz filter.

In order to avoid contamination with bacteria, 250 units/ml of penicillin and 100 units/ml of streptomycin were added to the cultures. The antibiotics in these concentrations do not impede multiplication of Lamblia, but indirectly promote its growth by being an additional nitrogen source for the fungi (Elinov, 1956).

Medium No. 1 was used to isolate the Lamblia, and medium No. 2 was used to maintain it. Medium No. 1 consisted of 25% inactivated human serum, 5% chicken embryo extract, 10% chicken amniotic fluid, and 60% Hanks' salt solution. Medium No. 2 consisted of 20% inactivated filtered human serum, 5% chicken embryo extract, 25% Hottinger's digest, and 50% Earle's salt solution. Five days after subculturing in medium No. 2, one ml of the medium contained 300,000 to 400,000 lamblias.

Usually the nutrient media are basic (pH 7.6-8.0) immediately after preparation. Carbon dioxide is filtered through the solution in order to lower the pH to 7.3-7.5 (for more details see Levi, 1957).

GLASSWARE

The quality and cleanliness of the glassware is very important since the lamblias multiply only after they attach to the walls of the flasks. Our flasks were the same as those used for insulin and penicillin. They were washed in boiling soda water, rinsed in warm water, and allowed to stand over night in a chromic mixture. After this, they were thoroughly washed with tap water and immersed for 12-16 hours in distilled water. The next day the flasks were again thoroughly rinsed in distilled water, and dried and sterilized with dry heat. The rubber stoppers were washed with a brush and soap, then boiled for 30 minutes in a solution of 5% sodium bicarbonate. They were next boiled for a total of 30 minutes in three changes of distilled water and sterilized in an autoclave.

PREPARATION OF THE MATERIAL FOR INOCULATION

The attempt was made to have sterile conditions while obtaining the duodenal contents for inoculation. It is important to mention that it is not possible to culture all of the lamblias obtained. Motile parasites, with well-defined, unimpaired structure and form, are the only ones suitable for inoculation, and lamblias which are obtained from patients receiving quinacrine are not suitable. Samples of duodenal contents were centrifuged and the sediments examined. In those that contained the fungi, the lamblias were usually more numerous and more viable, and maintained their motility for a long time. It was from this kind of sediment that we obtained our three cultures of Lamblia.

First the duodenal contents were centrifuged to remove the residue of bile and magnesium sulfate and concentrate the lamblias. Before centrifuging, the samples were diluted two-fold with Hanks' solution (pH 7.2-7.4) in order to reduce their viscosity. They were then centrifuged at 600-1000 r.p.m. for 10 minutes. After this length of time the majority of lamblias, with no marked change in their structure, appeared in the sediment. The supernatant fluid was drawn off and 2-3 ml of salt solution were added. The sediment was shaken up and again centrifuged.

This procedure was repeated three or four more times. After the last centrifugation, 1-2 ml of Hanks' solution were added to the sediment, and the latter was broken up and thoroughly mixed with the salt solution. Then 0.2-0.5 ml of the suspension were introduced into the flasks which contained chicken fibroblasts growing in medium No. 1. The flasks were then tightly plugged with rubber stoppers, placed on a slant (5-7° from the horizontal), and incubated at 37°.

CHARACTERISTICS OF GROWTH AND CULTIVATION

Three to four hours after inoculation the lamblias accumulated on the bottom of the flasks and attached to the fibroblasts. Growth of the protozoa was observed directly in the flasks under the low power of the microscope (objective 8X, ocular 10X). For the first two or three days the lamblias were adapting to the medium, and there was no marked increase in numbers. On the fourth and fifth days the lamblias began to multiply simultaneously with the fungi. The parasites appeared in pairs among the fibroblasts and then many of them bunched together (Fig. 1). Seven to 12 days later the chicken fibroblasts gradually began to break down, but the lamblias and fungi continued to grow.

On the second day after inoculation one ml of medium No. 1 was added to each flask. After this, 1/2 or 2/3 of the culture fluid had to be replaced daily and the sediment produced by the fungi drawn off.

The cultures were observed daily and, by gentle shaking, the sediment formed by the fungi was broken apart on the bottom of the flasks while the lamblias remained attached to the walls. The lamblias are difficult to observe 2 to 3 weeks after inoculation because a film of fatty material covers the walls of the flasks. This material is produced in the culture medium from lipoids and the fungi. It is better to observe the parasites after the medium has been changed, since the film usually disappears at this time. If it does not disappear, put in 2 to 3 drops of 96% alcohol. This concentration does not harm the lamblias and quickly dissolves the film. The film, as a rule, does not form in cultures with completed growth (Figs. 2,3).

It is always better to subculture when growth is complete and the number of lamblias in one ml of culture medium is not less than 100,000. Two to four hours after subculturing, half the culture medium was replaced with new. Before transferring, the cultures were vigorously shaken so that the lamblias were torn loose from the walls of the flasks and freed in the medium. 20,000 to 30,000 lamblia in one ml were introduced into each new flask. It is possible to add new medium to the old flasks and continue culturing the initial strain for a long time. We have cultures which are 7 months old and from which we have subcultured 15 to 20 times.

It was observed that a definite number of lamblias is found constantly in the supernatant fluid. We found 5,000 to 10,000 lamblias/ml of fluid, which increased to 30,000 to 40,000/ml. So it is possible to inoculate new flasks with the supernatant fluid and an equal volume of medium.

Various methods can be used to calculate the number of lamblias and fungi. The most exact figures are obtained with a hemocytometer. Some investigators may object to this method because shaking in the hemocytometer stops the growth of the parasites for 2 to 3 days.

The number of lamblias can be determined directly in the flasks. A scale of squares is made inside on the bottom of the flasks with a diamond needle, and the lamblias in the squares are counted daily.

The simplest way to calculate the number of lamblias is to use a microscope with an ocular scale and a fixed magnification. We used an 8X objective and a 10X ocular. Select a section that has the densest growth, and the intensity of growth can be traced for days in each flask and will give comparable data.

In order to study the morphological characteristics of the parasities and take photomicrographs, we vigorously shook the flasks and then introduced square cover glasses which had been treated with a chromic mixture. The lamblias accumulated on the cover glasses, attached and multiplied in the same way as on the walls of the flasks. Figures 4 to 7 are phase-contrast photomicrographs of lamblias on the cover glasses. They also show that we were successful in culturing the fungus Candida guilliermondi along with the lamblias. We attempted to culture the lamblias without the fungi, but did not succeed. We added an antifungi antibiotic, nystatin, to the medium. Within 24 hours the Candida had been killed and the lamblias were dying off. It is not known how the fungi benefit the lamblias. In the present case, it is possible to assume that the nystatin was active against the lamblias, but most likely the death of the fungi deprives the lamblias of some unknown factor of growth.

The biochemical and the culture characteristics of the yeast-like fungi have not been studied thoroughly; far more is known about the true yeasts. If it is assumed that there is a close resemblance between them, then it can be suggested that some of the biochemical and fermentation properties of true yeasts are inherent in the yeast-like fungi. It is known that the true yeast cells produce many reducing products. They are rich in co-carboxylase, carboxylase and vitamin B complexes. The yeast cells, breaking down with fermentation, release nitrogen and various metabolic products into the surrounding fluid. Sterols are accumulated by the yeasts when they are growing and secreted by the yeasts when they are disintegrating.

It is difficult to say which of the above metabolic products are the same for the yeast-like fungi and which of them are used by the lamblias. It is possible that the lamblias lack some fermentation systems and that they utilize the enzymes of the yeast-like cells to synthesize nutrient material.

Broader study of the relationship between the lamblias and the yeast-like fungi is necessary in order to discover answers to the questions about therapy for lambliosis and the use of antibiotics with some complex forms of diseases. At present we think it is possible to recommend the use of an antifungi antibiotic (particularly nystatin) along with atebrin in the treatment of lambliosis.

We think that with the aid of Lamblia cultures, it is possible to clarify some of the problems of morphology, physiology, and biology of the parasite, and in particular to decide the question of its pathogenicity and how to fight against it.

SUMMARY

1. Lamblia is fairly often found with yeast-like fungi of the genus Candida in the intestine of man.

2. Three cultures of Lamblia intestinalis with Candida guilliermondi were grown on chicken fibroblasts. The cultures of Lamblia did not develop without the yeast-like fungi. This bears out the synergistic relationship between these organisms.

3. The physiological and biochemical properties of Lamblia can be studied in culture.

FIGURE LEGENDS

Figure 1. A five-day culture of Lamblia on the wall of the flask. Those parasites seen in pairs are in the stage of division. In the center are seen accumulations of lamblias and below them are bunches of yeast-like fungi (magnification 8 X 10).

Figure 2. A phase contrast photomicrograph of Lamblia on a cover glass. In the center, growth of the parasites is complete (magnification 20 X 10).

Figure 3. A phase contrast photomicrograph of Lamblia on a cover glass where growth is complete (20 X 10).

Figure 4. Phase contrast photomicrograph of Lamblia and yeast-like fungi on a cover glass (magnification 40 X 10).

Figure 5. Phase contrast photomicrograph of Lamblia and yeast-like fungi on a cover glass (40 X 15).

Figure 6. Phase contrast photomicrograph of Lamblia on a cover glass in the stage of division (magnification 90 X 10).

Figure 7. Phase contrast photomicrograph of Lamblia. On the left is a Lamblia with four nuclei which is in the beginning stage of division (magnification 90 X 7).

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