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A Simplified Hyaluronic Acid Based Culture Medium for Mycobacteria Isolated from Human Lepromata [1]

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Skinsnes and co-workers (4) succeeded in culturing a mycobacterium, probably *M. leprae,* from human lepromata and from *M. leprae* infected mice. The media contained hyaluronic acid, sterile yeast extract solution and bovine serum albumin and glycerol. Incubation temperature was 37°C and maximal growth occurred in 21 days. In order to verify these findings, we inoculated the medium proposed by the Skinsnes group with bacilli isolated from six lepromata from lepromatous leprosy cases obtained from the Institute of Applied Leprology, Dakar, Senegal. Culture media inoculated with five aseptically removed lepromata became infected with banal saprophytic microorganisms even in the presence of 200 U/ml penicillin. Within ten days, acid-fast microorganisms were multiplying in the culture media inoculated with *M. leprae* from the sixth leproma. These cultures were easily subcultured in the homologous hyaluronic acid medium but failed to grow in Dubos, Middlebrook and Lowenstein media. Identification of these cultures is not yet available at the present time, but their cultural characteristics are similar to a culture provided by Skinsnes.

We then planned to study the respiratory mechanism, electron transport chain, energetics and other biochemical and biological properties of the cultures isolated by Skinsnes and ourselves from lepromatous leprosy cases. For studies, large amounts of cells are needed. Two problems were immediately confronted. On one hand, the obtained cultures produced a sub-optimal yield in the hyaluronic acid medium; and on the other hand, the commercially available hyaluronic acid, yeast extract solution and serum albumin are extremely expensive products, which represented an irrational drain on our research budget, because of the very large quantities of medium required for mass cultivation. Further, the preparation of the media is somewhat complicated due to the heat sensitivity of the products and the sterile filtering of the media involved in the preparation. We therefore immediately initiated systematic investigations directed at producing a simple, inexpensive culture medium; a modification which would provide high yield in the shortest possible time.

We are now able to present a simple culture medium which is easy to prepare, yields approximately eight times more cells in five days as compared to the medium used by the Skinsnes group. The medium is a simple umbilical cord extract supplemented with yeast extract powder and sterilized in an autoclave. To these, horse, bovine but preferably sheep serum is added. This medium is 86% less expensive than the original hyaluronic acid medium. No filter sterilization is needed thus simplifying and shortening considerably the preparation of the medium.

MATERIALS AND METHODS

Human umbilical cords were stored at –40°C. The frozen umbilical cords were thawed and washed with tap water to remove excess blood. Twenty grams of this tissue was cut with scissors into small pieces less than half an inch long. These were homogenized in a Waring blender with 50 ml 0.066M Na_2HPO_4-KH_2PO_4 buffer pH 7.5. Homogenization is usually completed within two minutes with 300 ml of the same buffer gradually added. The homogenized umbilical cord is then digested with 200 ml 1:250 trypsin (Sigma) for one hour at 34°C in a metabolic shaker.

The digested and now viscous umbilical cord solution was filtered through a nylon filter. A piece, longitudinally cut from ladies' nylon stocking, was stretched over a one liter beaker and fastened with a string. The filtration was accelerated by gently stirring the solution on the nylon filter with a spoon. The tissue fragments on the filter were transferred with a spoon into a beaker and washed three to four times with 100 ml KH_2PO_4,

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0.066M solution and filtered through the same nylon filter. The filtrate was then adjusted with KH_2PO_4, 0.66M solution to pH 6.0-6.2. The final volume should be one liter with a pH range between 6.0 to 6.2. This umbilical cord solution was autoclaved for ten minutes at 15 lbs. pressure and filtered through a Whatman filter paper while hot.

Yeast extract Difco 6 gm and glycerol 30 gm were added to the viscous opaque umbilical cord solution. When completely dissolved, the final volume was adjusted to one liter with the 0.066M phosphate buffer solution, pH 6.0.

This solution was then distributed in test tubes or flasks as desired. Nine milliliter amounts were distributed into 50 ml screw cap tubes or 90 ml into 500 ml flat culture flasks.

The medium was sterilized in an autoclave at 15 lb. pressure for 15 minutes.

The sterilized solutions were cooled to room temperature and then 10% sterile sheep serum was added under aseptic conditions.

Media were inoculated with minute amounts of the cultures isolated from lepromata or the reference culture (HI-75) obtained from Dr. Skinsnes. Two hundred units per ml penicillin G. sodium was added to the inoculated media.

Cultures were centrifuged and bacilli washed twice in saline solution. The washed bacilli were transferred into Dubos, Middlebrook and Lowenstein liquid and solid media.

RESULTS

Optimal growth was obtained when cultures were incubated at 33-34°C and when cultures were shaken ten cycles per minute on a shaker.

The growth rate was measured in a Klett spectrophotometer and the results are presented in Figure 1. Changes in optical density were plotted against time in days. The results show that after a short latency period, a rapid growth occurred in the umbilical cord medium. The maximal yield was reached with sheep serum added in 6 days as compared to 24 days in the LA-3. The yield was also considerably higher in the umbilical cord medium than in the LA-3.

Stained preparations were made at three day intervals from the cultures. The Ziehl-Neelsen technics modified by Skinsnes were used. In the hyaluronic acid medium, most of the bacilli remained non acid-fast up to the 14th day when gradually stronger and stronger acid-fastness developed. At the end of the third week, nearly all the bacilli showed definite acid-fastness. Bacilli were dispersed, appearing singularly or in small bundles and seldom in small clumps. In the umbilical cord medium, most of the cells were acid-fast after four days incubation, and at the sixth day nearly all the cells had the appearance of solidly stained acid-fast bacilli. Few cells appeared as solitary units or in small bundles. Most of the microorganisms were agglomerated into smaller or larger clumps having the typical arrangement found in globi as seen in the histological preparations or nasal smears of human lepromatous leprosy cases.

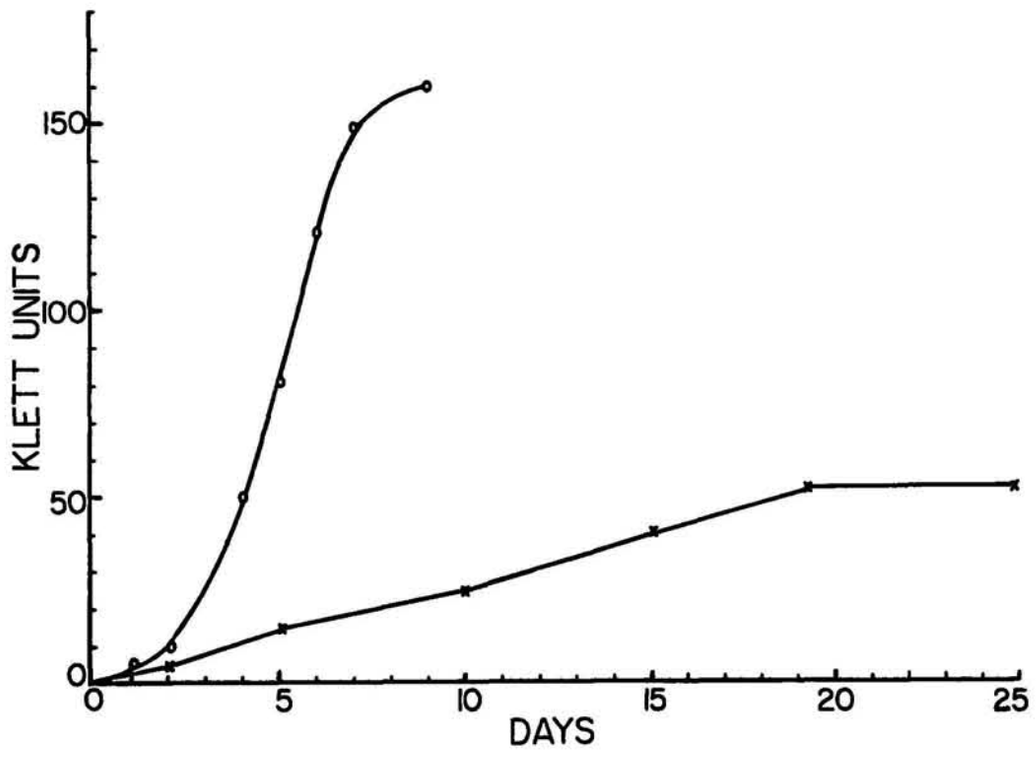

FIG. 1. Growth of acid-fast bacilli from a human leprotic nodule (LL-Dakar, Senegal) expressed in Klett units and plotted against time in days. Fifth, subculture in umbilical, glycerol, yeast extract, sheep serum medium o---------o. Fifth, subculture in hyaluronic acid, glycerol, yeast extract, serum albumin medium x---------x. Incubation temperature: 34°C.

Mycobacteria grown in these cultures did not grow on Dubos, Middlebrook or Lowenstein liquid and solid media, but were easily subcultured in the homologue umbilical cord or LA-3 media. When the cultures were transferred without washing into Dubos, Middlebrook and Lowenstein media, visible growth occurred in three to four weeks.

DISCUSSION

We have reason to believe that the reference cultures obtained from Dr. Skinsnes and the cultures isolated in our laboratories from a lepromatous leprosy case are hitherto noncultivated mycobacteria, probably *M. leprae*

(4). In previous communications, we presented experimental evidence that human and rat leprosy bacilli are metabolically competent microorganisms (1-3). Our results clearly showed that cells isolated from rat lepromata and from armadillos had a functional tricarboxylic acid cycle, a complete electron transport chain, and that these cells were able to oxidize some substrates which stimulated endogenous respiration. With appropriate substrates added, there was a measurable oxidative phosphorylation. We predicted in a previous paper that, "We would not be surprised if one day we would read a communication that *M. leprae* and *M. lepraemurium* are fast growing microorganisms" (2). By using the proposed umbilical cord, yeast extract, glycerol medium with sheep serum added, a really fast growing mycobacterium was cultured from human lepromata obtained from the Skinsnes group and from another isolated by our research team. The cultures are probably identical with *M. leprae.*

It should be noted that the umbilical cord extract contains hyaluronic acid and that sheep serum is rich in amino sugars, a constituent of hyaluronic acid. They substitute well for the commercially produced, expensive product and may present also other as yet unidentified substrates helpful to the growth of these mycobacteria.

SUMMARY

Acid-fast bacilli multiplied in liquid culture media containing hyaluronic acid when inoculated with mycobacteria from a lepromatous leprosy nodule. The culture was readily subcultured at ten day intervals in the homologue media, but failed to grow in the Dubos, Middlebrook and Lowenstein media. These findings confirm the results of Skinsnes *et al* (1975). Identification of this culture is not yet available, however it gives positive immunofluorescence with authentic anti-*M. leprae* serum. The obtained culture also grows as a chromogenic culture at 34°C on a simple medium prepared from trypsin digested human umbilical cord, yeast extract powder and glycerol. This medium can be sterilized in an autoclave, but filter sterilized sheep, bovine or horse serum must be added aseptically as an essential ingredient. The medium does not differ considerably from the hyaluronic acid medium proposed by Skinsnes *et al,* but it is easier to prepare, it is inexpensive and permits a logarithmic growth within seven days of the so far unidentified culture isolated from leprotic nodules.

RESUMEN

Se preparó una suspensión micobacteriana a partir de un leproma obtenido de un paciente con lepra lepromatosa. Con esta suspensión bacteriana se inoculó un medio líquido de cultivo conteniendo ácido hialurónico. Se logró un cultivo que pudo mantenerse por resiembras en el mismo medio a intervalos de 10 días pero que no creció en los medios de Dubos, Middlebrook y Lowenstein. Estos hallazgos confirman los resultados del grupo de Skinsnes (1975). Aún cuando no se ha logrado la identificación definitiva del microorganismo cultivado, se observó que el cultivo reacciona positivamente con un antisuero fluorescente dirigido contra el auténtico *M. leprae.* El microorganismo cultivado también crece en forma cromogénica cuando se cultiva a 34°C en un medio conteniendo extractor de cordón umbilical humano tratado con tripsina, extracto de levadura y glicerol. Este medio se esteriliza en el autoclave y se suplementa con suero de carnero, de bovino o de caballo, esterilizado por filtración. El suero adicionado asépticamente es un ingrediente esencial del medio de cultivo. El medio no es muy diferente del medio con ácido hialurónico diseñado por el grupo de Skinsnes pero resulta más fácil de preparar, es barato y permite, en siete días, un crecimiento logarítmico del microorganismo ácido-alcohol resistente aislado del leproma humano.

RÉSUMÉ

L'ensemencement en milieu à base d'acide hyaluronique d'une mycobactérie provenant d'un nodule lépreux lépromateux permet la multiplication de bacilles acid-fast. Bien que la culture ait subi plusieurs passages en milieu homologue à des intervalles de dix jours, aucune croissance n'a été obtenue sur les milieux classiques de Dubos, Middlebrook et Lowenstein. Ces données confirment les résultats de Skinsnes *et al* (1975). L'identification de cette culture est incomplète, toutefois avec le sérum anti-*M. leprae* elle montre une immunofluorescence positive. En milieu à base de cordon ombilical humain trypsinisé, d'extrait de levure et de glycérol la culture se révèle chromogène lorsque cultivé à 34°C. Ce milieu autoclavable renferme un ingrédient essentiel le sérum de mouton, de boeuf ou de cheval qui doit être préalablement stérilisé par filtration. De préparation relativement plus facile et peu coûteuse ce milieu diffère quelque peu de celui proposé par Skinsnes *et al;* et permet la croissance logarithmique en moins de sept jours de cette culture isolée de nodules lépromateux.

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