ATTEMPTS TO CULTIVATE *MYCOBACTERIUM LEPRAE MURIUM*

TEN YEARS WORK WITH NEGATIVE RESULTS

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The subcutaneous granuloma of rat leprosy is a typical lesion of the connective tissue. In the viscera, muscles, sense organs, genitalia or any other part of the body, lesions develop without exception preferably around veins and capillaries, not because blood circulation supplies the source of energy but because the vessels are embedded in connective tissue, a common feature wherever lesions occur.

Thus, in histologic sections bacilli are never seen in the parenchymal cells of the organs, but around the central vein of the liver, in the tubular organs in the submucous connective tissue. Bacilli never appear in the muscle fiber, but only in the interstitial tissue. In the central nervous system there are no lesions in the parenchyma, but only in the meninges and the connective tissue of the pia mater. Lesions are never seen in the peripheral nerves, but only in the region of the vessels of the epineurium. Furthermore, in the genitalia, spleen, and lymph nodes, lesions are found without exception in the connective tissue. This is an amazing and intriguing challenge for a working theory!

In our efforts to cultivate this parasite in connective-tissue cells, cultures remained sterile or at most only a limited growth was observed. Our working hypothesis has been that murine leprosy cells must take their energy from the environment in which they multiply abundantly, this being the connective tissue.

This working concept was formulated in 1952, at a time when advances in the chemistry and physiology of the connective tissue introduced revolutionary aspects into pathology. It seemed promising to search for energy sources among the components, building blocks and degradation products of connective tissue cells, in the ground substance and structurally related substances. Also included was a search for metabolites among the components of the inflammatory exudate-like fibrinogen and its polymers, and substances appearing in the inflammatory process.

We also planned to investigate energy utilization in the presence of added enzymes, considering the possibility that lack of energy utilization is due to lack of enzymes participating in the transformation of metabolites, or in the utilization of metabolites. For instance, D-glycosamine as a source of energy was tested in the presence of

yeast hexokinase, thus permitting the phosphatation of the D-glycosamine molecule. Similarly, fibrinogen was tested in the presence of thrombin or papain, fibrin with fibrinolysin, hyaluronic acid with hyaluronidase, collagen with collagenase. These substances were incorporated in the liquid and solid media. It was also necessary to perform all experiments at different temperatures, in different bases and at various ionic strengths and pH, the last being as low as pH 3.8 inside of some RES cells.

MATERIALS AND METHODS

Suspensions of *Mycobacterium leprae murium* from 12-week-old subcutaneous granulomas of rats were partially purified by repeated centrifugation in Hanks' balanced salt solution (BSS). Suspensions contained approximately 5×10^5 bacilli per cc., and 0.5 cc. was added to each tube of the media. Suspensions were freshly prepared for inoculations.

Each of the media was prepared in four variations. One was prepared with agar, one with gelatin, one contained bovine serum albumin in agar, and one had yeast supplement added to the agar. Forty μ/cc. of penicillin was added to each medium. BSS_{50} was used without exception in all samples of media, and the final pH was 7.2. Each variation of medium was prepared in 4 to 6 tubes, one-half of them incubated at 37°C and the other half at room temperature. In the few media that showed a limited initial growth, experiments were repeated with variations of ionic strength and pH. All samples were tested at the end of the 2nd, 4th and 10th weeks for the development of colonies, and for the staining properties and morphologic changes of the bacilli.

RESULTS

No growth was promoted in the presence of 10, 100 or 500 μg/cc. of the following substances:

1. Hyaluronic acid, crude umbilical cord extract, postassium hyaluronate, vitreous humor, or synovial fluid, with or without hyaluronidases of animal or microbial origin.
2. Chondroitin sulfate.
3. Acid-soluble collagen, alkali-soluble collagen, citrate-extracted collagen, of rat skin of young and adult rat-tail tendon; also the reconstituted collagens, with or without collagenase and gelatin.
4. Human and bovine fibrinogen, purified; intermediate polymers of fibrinogen; antihistamine- and 5-hydroxytryptamine-treated fibrinogen; fibrin obtained with thrombin and papain; fibrinolysin-treated fibrin.
5. Gastric mucin, submaxillary mucin, ovomucoid, crystalline egg albumin, seromucoid.
6. D-glycosamine and N-acetyl-D-glucosamine, with or without hexokinase; sodium glucuronate; glucuronic acid; β-phenyl-N-acetyl-D-glucosamine; β-benzyl-N-glucosamine.
7. Histamine bichlorhydrate, 5-hydroxytryptamine, thrombin, heparin.
8. Serum of heparin-treated rabbit; protamine sulfate; serum of protamine-sulfate-treated rabbit.
9. Mammary gland homogenizate of lactating rat; 3-0-p-D-galactopyranosyl-N-acetyl-D-glucosamine.
10. Neuraminic-acid, and neuramino-lactate, with or without purified neuraminidase.
11. Linear homoglycans: laminaran, amylose, *Torula* polysaccharide.
12. Non-linear (principally branched) homoglycans: 42 different dextrans with variations in molecular weight, structure, and origin.
13. Diheteroglycans: galactomannans from different vegetal origins, acid hydrolyzed galactomannans, d-galactose and d-mannose.

14. Triheteroglycan: slippery elm mucilage.
15. Tetraheteroglycan: gum arabic.
16. Esterified polysaccharides: carragenans, galactans.

Limited, insignificant, growths were obtained in the presence of 500 μg/cc. of heparin on simple agar containing BSS_{50} and 0.05 per cent serum albumin. In heparin-containing BSS_{50} with serum albumin 0.05 per cent added, a two- to three-fold multiplication was counted at 14 days. Although no further multiplication occurred after 2 weeks, the staining properties and the morphology of bacilli were particularly well preserved in the presence of heparin for over 3 months.

Similarly, small pinpoint microcolonies develop for not longer than 2 weeks on heparin-containing agar if a small filament of rat-tail tendon (from rats weighing not more than 40 gm.) is placed on the surface under aseptic conditions. Pinpoint microcolonies appear on the surface of the tendon and at a distance of ±2 mm. from it. A sterile zone can be seen immediately around the tendon, suggesting the presence of an inhibitor, and there is no growth at distances remote from the filament suggesting the slow diffusibility of a suspected macromolecule. There was no multiplication of bacilli in BSS_{50}-albumin liquid media in the presence of the tendon. Similar negative results were obtained with trypsin-treated tendon.

Although the past ten years have brought only daily disappointment resulting in 8,000 sterile tubes, further investigations are presently under way in these laboratories to explore the relation of lesions to the biosphere in which they occur: the pericapillary space of the connective tissue. Each element and building block, as well as molecules related in chemical structure or physical properties, to the components of connective tissue is under further investigation as a prospective source of energy for the Stefansky bacillus.

SUMMARY

The working hypothesis that has governed our work during the past decade is that the murine leprosy bacillus, the lesions of which are always associated with the connective tissues of the body, must take their energy from the connective-tissue environment.

In our culture work we employed a list of no less than 16 substances or classes of substances in various dilutions, with no promotion of growth.

In the presence of heparin, limited insignificant growths were obtained under certain circumstances. Growth effects around small filaments of rat-tail tendon suggest the presence of an inhibitor in the immediate neighborhood, and lack of growth at remote distances suggests the slow diffusion of a suspected macromolecule.

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ently under way in these laboratories to explore the relation of lesions to the biosphere in which they occur: the pericapillary space of the connective tissue. Each element and building block, as well as molecules related in chemical structure or physical properties, to the components of connective tissue is under further investigation as a prospective source of energy for the Stefansky bacillus.

RESUMEN

La hipotesis de trabajo que ha gobernado nuestro trabajo durante la década pasada es que el bacilo lepra murino, las lesiones del cual estan siempre asociados al tejido conectivo, debe tomar su energía del tejido conectivo que lo rodea. En nuestro trabajo de cultivos, hemos empleado una cantidad no menor de 16 substancias o clases de substancias en diversas soluciones, las cuales no provocaron crecimientos.

Con la presencia de heparina, fueron obtenidos crecimientos insignificantes en determinadas circunstancias. Los crecimientos alrededor de pequeños filamentos del tendon de la cola de la rata sugieren la presencia de un inhibidor en las vecindades inmediatas, y la falta de crecimiento a distancia remota, sugiere la difusion de una sospechada macromolecula.

Aunque los pasados diez años trajeron diarias desilusiones solamente que resultaron en 8,000 tubos esteriles, otras investigaciones estan actualmente en ejecución en estos laboratorios, con el objeto de explorar las relaciones de las lesiones con la bioesfera en las cuales ellas ocurren; el espacio pericapilar del tejido conectivo. Cada elemento y bloque edificante, como asi tambien las moléculas relacionadas en su estructura químicа o propiedades físicas con los componentes del tejido conectivo, estan en investigación como una fuente de energía posible para el bacilo Stefansky.

RESUMÉ

L'hypothèse de travail qui a présidé à nos recherches durant la décade écoulée est la suivante: le bacille de la lèpre murine, dont les lésions sont toujours associées avec les tissus conjonctifs de l'organisme, doit tirer son énergie des tissus conjonctifs environnants.

Pour nos essais de culture, nous avons utilisè pas moins de 16 substances, ou groupe de substances, en diverses concentrations, sans pouvoir entraîner une multiplication.

A l'occasion, une croissance limitée et insignifiante a été obtenue en présence d'héparine. Les aspects de la multiplication autour de petits filaments tendineux de queue de rat suggèrent la présence d'un inhibiteur dans le voisinage immédiat, et l'absence de multiplication à un niveau plus éloigné suggère la diffusion lente d'une macromolécule suspecte.

Quoique les dix dernières années n'aient été qu'une déception quotidienne, avec 8,000 tubes stériles, la poursuite des recherches est en cours dans ces laboratoires, dans le but d'explorer la relation des lésions avec la biosphère où elle se produisent: l'espace pericapillaire des tissus conjonctifs.

Chaque élément, chaque bloc constitutif du tissu conjonctif, ainsi que les molécules qui leur sont apparentées au point de vue des propriétés chimiques ou physiques, fait l'objet de recherches, afin de définir leur rôle éventuel comme source d'energie pour le bacille de Stefansky.