Studies upon Leprosy

II. ATTEMPTS TO CULTIVATE THE MYCOBACTERIUM LEPRAE.
(COCCHOTHRIX LEPRAE, LUTZ 1886). ISOLATING AN ACTINOMYCES FROM A
LEPROMA. THE ACTINOMYCES Lepromatis n. sp. (HILDA’s sample) (1)

BY

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(With plates 40—43).

In the course of this year, I performed several experiments with a view to obtain cultures of the germ of leprosy in artificial media, all of them having proved negative. As a rule the media, smeared with emulsions of leproma, extracted with skin, are always contaminated within 24 hours.

The female patient H. S., who was recommended to me by Dr. OSWINO PENNA, provided me with material, in which HANSEN’s bacilli were profuse, consequently perfectly suitable to cultures and inoculations. On the 5th of July, a. c. I extracted from her left arm, with a bistoury, a large flourished leproma (fig. 1 a) which I triturated in a sterilized mortar, with a saline solution and had thus a copious emulsion prepared of acid-alcohol-fast bacilli, in which no other germ was likely to exist. With such material, I inoculated several small animals, and effected smearings on common media. On the following morning, I noted a rich proliferation of germs which occurs, as a rule, in any medium. Two days later, (7th of July) I extirpated from the right fore-arm of the same patient, another large leproma (Fig. N. 1, b). I proceeded in another way, which might as well be named enucleation of the leproma: I had the skin cut open crosswise, detached the four corners of same, leaving thus the leproma uncovered, of a yellowish appearance, and easily detachable. I withdrew it without any skin, triturated it by the same process, thus obtaining a homogenous emulsion, very rich in bacilli, and, with it, inoculated animals, repeating the smearing in the various cultural media.

(1) See: Supplement n°. 4 (Dec. 1928) of the “Memorias do Instituto Oswaldo Cruz”.

Other lepromas, extracted in the same way, were divided into tiny fragments, which were laid on glycerine agar, in slanting surface, and on glycerine potato.

Twenty-four hours later, none of the tubes showed any contamination. And so on, during the following days, which caused me a great pleasure. Most of the tubes had been kept at the room temperature.

The macroscopic examination of these cultures did not show anything noteworthy, up to the 15th day, past which, a smearing in a glycerine broth, began to show a slight clot in the bottom of the tube. The medium kept limpid. On the 23rd day after smearing, preparations of this broth, gathered with a pipette at the bottom of the tube, and stained by ZIEHL-NEELSEN, revealed a mixed culture of acid-alcohol-fast bacilli in bundles or masses (fig. 2) and filaments alternately ramified, impregnated with methylene blue.

During this period, Dr. LUTZ and Dr. FONTES examined the culture and saw the smears, judging them interesting.

On the 3rd of August, being the culture still richer in filaments, and always retaining masses of bacilli of leprosy, it was transferred to agar, glycerine agar, glycerine potato, plain broth and glycerine-broth.

On the 6th and 11th, new sub-cultures were made, two of which by Dr. A. MACHADO. The sub-cultures showed quickly a fungus, whose macro and microscopic appearance was that of a typical actinomycetes, as declared by Dr. O. DA FONSECA, Chief of the Section of Mycology of this Institute, who has been kind enough to accompany my researches and collaborate in the description of the fungus.

A few days later, the tube containing this original culture was accidentally broken by my laboratory assistant.

Examining then the sub-cultures in plain broth, and the liquid of the potato culture, I verified to exist here also, intermixed with the filaments of the actinomycetes, bundles of acid-alcohol-fast bacilli.

All the sub-cultures germinated luxuriantly, some of which soon within the 24 first hours, always pure, i.e. containing the same actinomycetes as will be described hereafter.

Having laid fragments of leproma in tubes of glycerine agar, I noticed that the HANSEN's bacilli multiplied largely within the same leprous tissue, without, notwithstanding, invading the culture media, which would account for further replantings having remained unsuccessful.

Some of this material being gathered after 60 and up to 120 days, by means of the platinum loop, proved to be rich in acid-alcohol-fast bacilli, as shown in fig. 4, B and C of the coloured plate and the photomicrograph N. 4.

This special feeding stuff being exhausted, the culture stops developing and cannot be regenerated any more. This I verified, not only with the material from the patient H. S. as also with that of three more others, which is but a more repetition of NEISSER's, BEZANCON's, EMILE—WEIL's, and NICOLLE's investigations, if referring to ancient authors, and, among modern searchers, HERMANN von SCHROETTER's of Vienna, with his smearings in broth with 2/3 of leper serum, SUSVIOLA GUARCH of Montevideo, in CARREL's medium, in which I saw lepromas kept in culture for 11 months, with luxuriant proliferation of the bacilli of leprosy, without the attempted adaptation to the artificial medium. What I viewed in Osaka (Japan), in HARADA and KUJO's laboratories is of still higher meaning: the fragments of leproma serve as a natural food for the bacilli, which, always growing in number, adapt little by little to the solid.
part of the HARADA’s medium, later on to the fluid part, allowing then replantings and inoculations to laboratory animals.

Transplanting those fragments of leproma from an exhausted to new media, after 105 days of smearing, did not prove anything available for the intended isolation and culture of the HANSEN’s bacillus.

Acid-fast bacilli are found here during a long time, still rather most probably have their origin in the disaggregation of the leprous tissue.

MORPHOLOGY OF THE ACTINOMYCES LEPROMATIS.

CULTURES IN FLUID MEDIA:

1) Plain broth. 5 days (from the plain broth culture of 7 Aug. 1928). Spheric white colonies, appearing like cotton-wool, darker in the middle, measuring from 1 to 3 mm in diameter, sunk to the bottom of the tube. The medium was limpid and of a normal colour.

After 10 days, at the room temperature, the colonies show the same aspect, forming however a heavier deposit. The medium keeps still limpid, but slightly pigmented. Now and then, the colonies develop themselves amidst the liquid, without forming either a deposit or cloudy veil, as also without sticking to the glass of the tube.

After 70 days, the colonies continue isolated, more or less spheric or disfigured at the bottom of the tube. The medium remains still clear, nevertheless, it is impregnated with a brownish pigment. One may also observe the development of a collar on the wall of the tube, at the top of the medium, whose lower part shows an irregular aspect, of a wax-like yellow shade. The upper part appears sporulated, covered with a pulvulrent coating of cretaceous aspect.

With 90 days, the colonies cease to be globulous, and appear like a heavy clot, of cotton-wool like aspect. In a tube with 82 days, there was a formation of a sharp cone-shaped yellow colony, on the surface of the liquid.

A tube, smeared on the 10th October, starting from plain broth of 21st August, shows a ring of large globulous colonies, in the middle part of the broth. Looking upwards, the liquid is strongly pigmented. In the lower part of the broth, there are small floating colonies, no deposit, and very little pigmentation. At the top of the medium, sticking to the glass of the tube, there are, flat white and radiated colonies.

2) Plain broth covered with a coating of paraffin oil. The 5 days culture shows globulous white colonies, sunk to the bottom of the tube, macro and microscopically similar to those of the plain broth without oil, smeared on the same day. The case is therefore, of a facultative anaerobic actinomyces.

The medium remains clear and without pigment.

After the 10th day, growth has taken little increase. The medium is slightly pigmented. After 20 days, there has been no further change.

Another 10 days culture, starting from glycerine potato in water, of first replanting, produced small globulous colonies at the bottom of the tube, and the fluid remained clear without pigmentation.

3) Glycerine broth at 5%. Cultures of 5 and 10 days showed colonies of a like appearance as those with simple broth, however in smaller number and with less luxuriance. On the 10th day, there is a slight pigment. On the 70th day, the medium is still limpid, with a brownish pigmentation, less marked than in plain broth. There is, at the top, no ring being formed. Culture of 10 days, starting from glycerine potato in water, of 7th August, turned the medium slightly
cloudy, producing an amorphous deposit, similar to cotton-wool flocks.

4). Milk. Culture of 10 days. No coagulation of the medium. The culture shows the appearance of a veil extending from up to downwards, along the glass, in the shape of a ring, where the back part of the colonies may be observed with their bright yellow coloration in several points, brownish yellow in some others. The veil appears with an orange colour and allows easily to be fragmented. The lower half of the milk shows normal aspect and colour. The coagulation of the milk took place on the 15th day. This coagulum is separated from the culture, the latter being superficial, by a layer of water clear serum. With 25 days, the coagulum appears pigmented in brown at the top being separated from the superficial culture by a layer of a one centimetre high water-like fluid. The culture that covers the medium is of a dark colour and measures 4 mm. in height. Looking upwards, there exist, sticking to the glass, ferruginous, dry fragmented colonies, which aspect remains almost unchanged up to the 40th day.

5. Water with 1% pepton and 1% leper serum. Culture of 5 days shows profuse, globulous colonies at the bottom of the tube, of exceedingly variable size, some of which having up to 3 mm. diameter. The medium is slightly impregnated with brownish pigment. On the 10th day, it shows many superficial colonies sticking to the tube, of various size, with a lichen-like aspect, with white zones (sporulation) in the borders and brownish shade in the middle. The medium keeps unchangedly clear, but strongly pigmented. On the 20th day it showed a superficial ring of white pulverulent colonies of from 2 to 3 mm., sticking to the tube. The back part of these colonies is coloured in brown. On the 35th day, these white colonies appear larger, dryer and the liquid is strongly pigmented in brown, without increase of the deposit.

CULTURES IN SOLID MEDIA

1) Slanting agar, plain. Cultures of five days, starting from the original in glycerine broth, showed translucent colonies, greyish, strongly sticking to the substratum, with a rather slightly sharpened medium zone, from whose centre, 4 to 6 star-like depressions or wrinkles start, sometimes disposed crosswise (Fig. 5 a and 2 of the colored plate).

These same cultures, with more than 60 days, showed large greyish or yellowish colonies, with a flat and plain periphery of circa one millimetre width. The adherence to the medium is much accentuated.

The first replantings on slanting plain agar resulted in confluent, scarcely isolated colonies, forming an almost uninterrupted rough coating, showing a striking resemblance with sample N. 39 (Lieske's book: Strahlenpilze) of the aerobic actinomyces isolated from the blood of a man suffering from actinomycosis, and cultivated in peptonized extract of beef with agar.

Sub-cultures of five days, starting from glycerine agar, preserved at the laboratory temperature, show colonies with more or less 3 millimetres diameter, sharpened, very often of a white shade in their apex, in which a dark point may be observed, corresponding to a small hole; after ten days, the colonies measure about half centimetre in diameter, and are of a wax-like color, with a very rough, almost cerebriform area.

On the 20th day, they show a more or less uninterrupted rough coating, similar to a varnished surface, translucent, almost greyish. The colonies growing in the neighbourhood of condensation water are isolated, of from two to three millimetres in diameter, coniform, depressed
in the vertex, where from two or three radiated wrinkles start.

After 60 days, the culture presents the form of a more or less continuous, rough and yellow coating, specially in the borders, in which sporulation may appear. The medium shows impregnated with a brownish pigment. The back part of the colonies is slightly orange.

After 60 and a few more days, the medium appears to be exhausted and the culture begins to degenerate.

Smearings on PETRI's plates, starting from the glycerine agar, show on the 5th day, larger colonies than in tubes; these are white, of pulverulent aspect (fig. 1, colour plate) and more or less nummular shape, when coalescent.

Around each colony, one may see a halo of circa 1 cm. diametre, of brownish pigmentation, which goes on spreading out towards the periphery. The back part of the colonies is slightly orange.

Cultures starting from plain agar, of 2nd generation, keep always their greyish translucent coating, and do not sporulate up to the 90th day, while those starting from glycerine agar, result in conform confluent and yellowish colonies; their utmost development takes place up to the 20th day, after which it keeps stationary, regressing only with difficulty.

2) Glycerine agar. Cultures of 10 days, starting from the glycerine agar at 5% on slanting surface show in the area of the medium, a thick, continuous and rather rugged coating of a gold yellow colour, with a tendency toward orange shade. The medium begins to get pigmented. Sometimes at this stage, the formation of zones of sporulation starts. On the 25th day, the orange coloration is more accentuated, as also the pigment impregnation.

After two months, the culture consists of a thick, irregular, rugged coating, full of protruding zones, almost cerebriform and of a gold or orange coloration. The medium is impregnated with brown pigment. At this stage, the cretaceous white coating, which corresponds to zones of sporulation, has invaded a great part of the culture (fig. 5 b and c).

We have already noted that in plain gelose on a PETRI's dish, this sporulation is a very early one, perhaps owing to a better condition of aeration of the cultures. Under different conditions, this sporulation, in weak nourishing media, occurs also very early, in little vigorous colonies, seeming as struggling against dysgenetic conditions.

Stick cultures on agar, develop more slowly; but after two weeks already, the brownish pigment has grown up to 1 cm. deep into the medium. Superficially, the colonies are absolutely like those above-mentioned. The back of the colonies developed on the glass side of the tube may distinctly be observed.

30 days culture, starting from plain broth, of July 7th (2nd generation) shows large radiated cones-shaped colonies. These are confluent, yellowish and measure half centimetre in diametre. No sporulation noticeable, and the medium is spotted with a uniform light brown pigment. The last sub-culture showed a harder germination, perhaps owing to the composition of the medium. Fig. 6 (a and b) show old culture—3 months—in glycerine agar.

3) Butter Agar. Five days slanting cultures, starting from glycerine agar, developed with full vigour, in a continuous coating of coalescent, rather translucent colonies, of yellowish shade, without macroscopically visible sporulation regions.

The reverse side of the colonies, which developed on the wall of the tube, are of a bright yellow shade. Brownish pigmentation of the medium little accentuated.

On the 10th day of germination, the culture had reached the whole superficial extent of the medium, whose pig-
mentation increased a little, no sporula-
cohol-fast, as advised by LIESKE. On the
20th day, the culture covers the whole
area, in a yellow, continuous and rugous
layer.

4) Olive Oil-Agar. Culture of the
above mentioned make, showed on the
5th day, an aspect similar to the fore-
going example, though the colonies were
more isolated, less coalescent, with a dar-
ker yellow colour tending toward orange.

The pigmentation of the medium is
greater than on butter agar. On the 10th
day, the culture turned more vigorous,
with a slightly increasing dark pigmen-
tation. No noticeable sporulation is seen
macroscopically. The cultures on butter
agar and olive oil-agar were purposely
to transform the actinomyces into acid-
olcohol-fast, as advised by LIESKE. On the
20th day, this culture shows a difference
with the foregoing, by the fact of ap-
pearing slight glittering, leaving an im-
pression of being wet. No sporulation
noticeable. In the 2nd generation, in tu-
bes with 22 days, we found acid-fast ba-
cilli. Neither here, a sporulation is seen.

5) Gelatine. A 20 days culture pro-
duced small colonies adhering to the
tube, on the area of the medium, co-
vered with sporulation. There was no
liquefaction of the medium.

6) In Sabouraud’s medium, there was
but a poor proliferation of the actinomy-
ces, almost equal to nothing.

7) Glycerine potato. Luxuriant and
quick developing in the form of coales-
cent colonies, of cerebriform aspect,
orange yellow coloration (specially in the
middle) besprinkled with numerous grey-
ish white spots, of cretaceous appear-
ance, which correspond to sporulation zo-
nes. The medium is invaded by a violet
blue, rather black pigmentation, which
at times spreads out until within the very
group of the colonies.

The condensation water appears quite
clear, without any noticeable germina-
tion.

After 15 days, the appearance re-
mains unchanged, simply the medium hav-
ing grown darker, and the colonies show-
ing taller and more coloured. Sporula-
tion exists in the whole culture (fig. 7 a).

The fluid is still clear, though show-
ing a white veil.

On the 25th day, there was no tan-
gible alteration.

The development of the culture had
reached its maximum point on the 15th
day. Sporulation is more accentuated on
the borders. In one of the tubes, the
condensation water got pigmented, owing
to the proliferation of a few spherical
colonies, similar to those observed in
broth.

8) Petroff’s medium. Very slow de-
velopment during 10 days. On the 15th
day, one of the tubes showed a pretty
colony, rather outbudding of 2 mm. in
diametre against 1 1/2 in height, of a
brisk yellow coloration. The reverse of
the colonies show an almost quite black
pigmented halo, encircling them very
tightly. Up to the 25th day, no increase
of the colonies has taken place, but the
former ones (centre of the medium) are
seen very luxuriant and of cerebriform
aspect.

9) Loeffler’s serum. 10 days replant-
ing, from glycerine agar, showing colo-
nies of different appearances according
to the region in the tube.

The colonies which developed in con-
tact with condensation water show a co-
ne-shaped aspect, with a depressed or
umbilicated central part, with bright
yellow shade. The periphery of same
shows a series of radiated wrinkles, and
is of a greyish yellow shade. Around these
colonies, a dark brown, rather black halo
develops, forming a crown of more or
less half a centimetre height. The back
side of these colonies shows, inasmuch may be appreciated, a very dark colora-
tion.

The colonies developed in the middle point of medium, of a lighter orange yellow than those described above, are cone-shaped and have an irregular, almost cerebriform area. Their reverse has the same shade as the front part.

The colonies which developed themselves within the dryest part of the me-
dium, are in appearance alike to others. Nevertheless, they are almost flat, and provoke a dark brown pigmentation. The dryest ones consist of excentric zones, very often covered with a cretaceous layer.

On the 15th day, the colonies in contact with condensation water had grown much more. Those in the dry zone of the middle part were atrophied, the dark pigmentation of the medium being more accentuated. The condensation water is quite clear, but with pig-
mentation.

Up to the 25th day, there was no change. On the 40th day, a part of the culture,—the upper,—was degenerated, the medium being exhausted.

Three or four months cultures, when replanted, germed normally, showing two months later, the same characters as described hereabove.

MICROSCOPICAL EXAMINATION.

The Actinomyces lepromatis, like most of the actinomycces, is Gram-posi-
tive and not acid-alcohol-fast. Its passage through media with butter and olive oil originated acid-fast bacilli. A new smearing of same, performed after months, did not give the same results; the acid-fast elements were filaments, but not bacilli.

Smearings of cultures in all the usual media, even in plain agar, on slanting surface, in which case its colonies stick

very strongly to the substractum, show always this fungus with its characteris-
tical morphology:—rudimentary thallus, colourless (when examined unstained be-
 tween plate and laminula) with genuine ramifications disposed alternately (fig. 8). These long filaments never appear in bacillar fragments in usual media. It belongs thus to the first type of the Actinomycetaceae.

Stained by the Gram’s process, being the culture flourishing, all or al-
most the whole of the filaments appear plainly with a dark violet shade. Older cultures show filaments which the Gram does not easily stain.

When dyed by ZIEHL-NEELSEN’s method, as well the new as the older cultures show only filaments plainly stained in blue. The case is then of a sporulous aerobic species.

As a rule, sporulation begins in the middle of the culture under the shape of a superficial whitish coating, of cre-
taceous aspect, easily detachable with the platinum loop, with more facility than the colonies. Smearings of this spo-
rulation, when stained by Gram, show masses of coccoid elements, slightly elon-
gated, with the appearance of minime seeds (Fig. 9). The spores are Gram-po-
sitive.

Smearings of cultures in agar butter (2nd, 3rd and 4th generations, respecti-
vively with 22, 4 and 10 days) showed fields with filaments equal to those of fig. 8, with fragmented filaments in non acid-fast rods (fig. 10), leaving the impression of a lysis of the mycelium with preservation of its regenerating elements, and also fields with filaments, rods, and masses of bacilli, all plainly acid-alco-
hol-fast. (fig. 11 and 12 and colour plate 5, B).

Such fact is a proof that the Actino-
mymes lepromatis may turn into the form of, acid-alcohol-fast bacillus, by absorp-
tion of the lipoids of the medium, a
property which perhaps may become permanent, by successive passages through the same medium, which we, however, till now, did not succeed to obtain.

Studies are further carried on, not only in respect to the biology of the new fungus, as also to its experimental part. Inoculation of same, in small laboratory animals, determined death in these after only a few days, and, only, one Guinea pig, inoculated intraperitoneally, gave me an opportunity to observe very thin and scarce filaments, in smearing of its viscera. Inoculations in the mamma of a goat and a bitch, did not provoke any lesion after 6 months. Three rabbits inoculated intravenously died without showing either the lesions or the parasite.

CONCLUSIONS

1. I do not know yet the relationship between the Actinomyces lepromatis and leprosy. Its being isolated, is the partial repetition of the results of DEYCKE and RESCHAD’s, WILLIAMS’, ROST’s, KEDROWSKI’s, HERMANN de SCHROETTER’s and others’ researches.

2. Prof. W. J. KEDROWSKI, of Moscow, after long years studying, arrived at the conclusions that the bacilli of leprosy and of tuberculosis belong to a group of actinomyces.

3. Recently KEDROWSKI, BRULOWA, PLATONOV, etc., succeeded in getting the mutation of old cultures of bacilli of leprosy and of tuberculosis into actinomyces.


Manguinhos, 31st December, 1928.