

A NONCHROMOGENIC CULTURE OF AN ACID-FAST
BACILLUS ISOLATED FROM THE NASAL MUCUS
OF A LEPROSY PATIENT;
ITS VIRULENCE FOR LABORATORY ANIMALS¹

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Relatively few attempts have been made to isolate and cultivate Hansen's bacillus from the nasal mucus of leprosy patients. Between 1928 and 1950 I tried many times, unsuccessfully, to obtain such cultures. Only recently have I been able to get from that source a strain of an acid-fast bacillus, which is described in this report.

The patient was a white girl (Dalva R.), 20 years old, born and living near the Instituto Oswaldo Cruz, who came to my laboratory for a pre-nuptial examination on December 13th, 1950. She was to be married on December 30th. There were presented a generalized lepromatous infiltration of the face, ears and knees; cyanosis and edema of the hands and feet; widespread roseoliform spots on the thighs, and nodules on the legs. Clinically she was a L3 case. Smears of the nasal mucus, taken from both sides with a curette, and of the lymph from the ears—and also a biopsy of the left knee—were strongly positive, showing very many large globi of coccoid bacilli.

BACTERIOLOGY

Because the patient had not been subjected to any anti-leprosy treatment and the nasal mucus was so rich in bacilli, as much material as possible was taken, treated by Petroff's method, and inoculated onto 21 tubes of Loewenstein's medium. After incubation at 37°C. for 37 days, one tube of the 21 was found (on January 20, 1951) to have five pin-head sized white colonies. The other tubes remained sterile for 30 more days, when they were discarded.

Ten days after the colonies were first observed, the culture presented a thin, creamy layer with three round, lentil-sized colonies in its center. Smears from one of those colonies stained by the Gram and Ziehl-Neelsen methods proved that the culture was a pure one of an acid-fast and gram-positive bacillus. The culture was then transplanted into four tubes of Loewenstein's

¹ Summarized from a paper read on December 14, 1951, at the Third Pan-American Leprosy Conference, Buenos Aires, Argentina.

medium and one of 5 per cent glycerin agar. On February 15, 1951, this culture (second generation) was registered in the collection of mycobacteria of the Instituto Oswaldo Cruz under the designation of Strain "Dalva" (Souza-Araujo, 1951). On the same day Dr. Laerte de Andrade proved that this new culture was as strongly fluorescent (2-plus), and as strongly Dubos-positive (2-plus) as the "Vallée" strain of *Mycobacterium tuberculosis*.

Macroscopic description.—Reexamination on November 20, 1951, of the original culture on Loewenstein's medium—then 10 months old, kept at room temperature for 8 months of that time—showed a thin layer of growth, strongly adherent to the medium, grayish-white in color, dry and powdery in appearance, covering the entire surface of the medium and extending for 1 to 2 mm. onto the wall of the tube. The second generation on this medium showed the same aspect as the original one, but with more exuberant growth in the bottom of the tubes. On glycerin agar the growth was slower, but the culture became eugonic (R type), the colonies being round, warty, dry, easily removable with the platinum needle. Tubes of Loewenstein's medium in the second and third generations, from 3 to 7 months of age, showed a thin granular growth of sandy aspect, with some verrucous, eugonic, pale-rose colonies (mutation to pink?). The tubes with warty colonies were similar to certain strains of the tubercle bacillus. On Dorset's medium without glycerin the germination was very poor, as it also was on glycerin potato. The culture does not liquefy gelatin. In 5 per cent glycerin-broth it grows only in the bottoms of the tubes, producing round free colonies which after shaking have a pearl-like appearance. The medium remains quite liquid and transparent. No trace of veil formation has been seen as yet. The best media for the growth of this bacillus are Loewenstein's and glycerin agar.

Microscopic description.—Smears of cultures 70 days old stained by the Ziehl-Neelsen method showed mostly coccobacilli, strongly acid-fast, and a few bacillary forms with one, two or more granules. There were many free metachromatic granules throughout the smear. Smears of the colonies grown in glycerin broth (third generation) show great masses of granulated elements, like gigantic globi, and also some dark red bacilli in a cloudy-rose symplasm.

Stained by Gram's method, all of the elements described are

seen to be strongly gram-positive. Smears stained by Fontes' method, which is the most suitable one for the study of the morphology of the mycobacteria, are the most typical and beautiful. Smears of the deposit of a glycerin broth culture, three months old, stained by this method showed pale-rose bacilli, of medium size, all of them granulated, the granules dark blue in color. There were sometimes one large central nodule, or two of them in bipolar location, or many granules disposed as a rosary; there also were free, widely distributed granules and masses of granules simulating large globi. Staining by this method smears of the R type culture on glycerin agar, 7 months old, produced beautiful preparations, surpassed only by smears of fresh lepromas stained by the same method. The bacillary forms, pale-rose in color with dark-blue nodules, were prevalent.

INOCULATIONS

Black mice.—On August 13, five black mice (American race) were inoculated each with 0.5 cc. of an emulsion of this culture grown on Loewenstein's medium, the inoculations made subcutaneously in the right groin. Eight days later, three of these mice showed inguinal nodules the size of a grain of corn. After another three weeks (August 30th) these three mice were killed. Two showed musculo-cutaneous nodules and one a skin tumor. All nodules when opened gave forth purulent material rich in acid-fast bacilli, mostly in mononuclear phagocytes which were filled with the bacilli. There were masses of bacilli simulating globi, and also some gigantic, granulated and vacuolated bacilli such as have been seen in experimental inoculations with the "Chaves" strain. The lymph nodes and spleens of these animals were also rich in bacilli. Cultures of the pus were made, without result.

The histopathology of the nodules of two of the mice was reported by Dr. Rita Cardoso (P.C. 17,360) as follows:

(1) Hypodermic abscess, rich in acid-fast bacilli. Around the abscess there is proliferation of fibrocytes, starting the formation of a capsule. In the adjacent dermis there is infiltration of mononuclear cells. The abscess penetrates into the dermis at one point. (2) Same structure as the first specimen, showing great numbers of acid-fast bacilli in the cytoplasm of vacuolated mononuclear cells, observed inside the dermis.

The other two mice, apparently in good health, were killed three months after inoculation. No nodules were found. Smears of material scraped from the inner surface of the skin of the

groin, from the lymph nodes, and from the spleens were positive for acid-fast bacilli, but the other organs were negative.

Guinea-pigs.—An August 17, two guinea-pigs were inoculated, each with 1 cc. of an emulsion of the second generation of the culture, in the groin. One week later one of them showed at the point of inoculation a hard nodule the size of a hazel nut. In the second week both animals had nodules, which in the third week became smaller. One of these guinea-pigs was killed on September 6, three weeks after inoculation. Necropsy showed an olive-sized subcutaneous nodule, with a central abscess the pus of which was very rich in acid-fast bacilli grouped in globoid masses, intra- and extracellular. Sowed onto Loewenstein's medium, this pus gave no retroculture.

The histopathology of this guinea-pig was reported by Dr. Rita Cardoso (P.C. 17,383) as follows:

Tumor: Sections of the tumor show an abscess with abundant acid-fast bacilli in the cytoplasm of polymorphonuclear leucocytes. In the capsule of the abscess there are mononuclear cells filled with such bacilli, and also a discontinuous infiltration of the neighboring connective tissue by mononuclear cells. *Lymph nodes:* The lymphatic sinuses are dilated and contain large vacuolated cells filled with acid-fast bacilli. *Lung:* Atelectasis, with foci of hemorrhage and, in the neighboring alveoli, the presence of large mononuclear cells, their cytoplasm vacuolated and filled with acid-fast bacilli. *Kidney:* In the submucosa of the *bacynete* are seen foci of infiltration by large mononuclear cells with acid-fast bacilli, and also lymphocytes. Mononuclear cells with acid-fast bacilli are also seen in some of the glomeruli. *Liver:* A few cells with acid-fast bacilli are seen in the sinusoids. The hepatic parenchyma shows no significant lesions. *Spleen:* In the blood vessels and lymphatic sinuses acid-fast bacilli are seen.

In this report there is nothing suggestive of tuberculous lesions.

Cebus fattuellus.—On August 17 one cebus monkey was inoculated in the forehead and cheeks with 1.5 cc. of an emulsion of this culture. This monkey is black and hairy, and consequently the observation of skin lesions is difficult. One week later, at each point of inoculation, there was a small, bean-sized nodule, which remained unchanged for more than another week. Three weeks after the inoculation the animal presented total alopecia around the face. On October 15th, at which time all of the nodules had regressed slowly, the largest of them, on the right cheek, presented a leishmaniasis-like ulceration. Biopsy of one such nodule showed that it was reduced to an abscess the pus of which was rich in acid-fast bacilli, isolated and in globi, all of bacillary form, none coccoid.

This animal was reinoculated twice with small doses (0.5 cc. each) of the culture suspension. Two bacteriological examinations of the feces gave negative results. On November 14th another nodule, a secondary one, was biopsied. It showed a regressive purulent nodule, the pus containing many acid-fast bacilli of medium size. The animal, which was cured of its alopecia, is being kept under observation.

SUMMARY AND CONCLUSIONS

1. The isolation and cultivation, from the nasal mucus of a leprosy patient, of an acid-fast bacillus virulent for laboratory animals is described.

2. The culture, called the "Dalva" strain, is nonchromogenic and of the R type. It is strongly positive to the Dubos test, as strongly as the Koch bacillus, but it does not produce tuberculous lesions in guinea-pigs.

3. In guinea-pigs and in black mice this culture caused general infection and considerable lesions, the histopathology of which is recorded.

4. In the original culture on Loewenstein's medium the germs were predominantly coccobacillary, becoming mostly bacillary in the lesions of the three species of laboratory animals inoculated, guinea-pigs, black mice and the cebus monkey. The culture has not yet been recovered from experimental lesions.

5. This strain is acid-alcohol-acetone-fast, gram-positive, and strongly fluorescent. Stained by the Fontes method it shows a morphology similar to that of the Hansen bacillus in fresh leproma emulsions.

6. It being so difficult to get cultures of acid-fast bacilli from leprosy lesions, especially from the nasal mucus, it is suggested that systematic attempts be made to obtain new strains from the nasal mucus of all lepromatous cases interned in leprosy colonies.

ADDENDUM

In further experimentation since this report was prepared, the cebus monkey was reinoculated on Jan. 15, 1952, subcutaneously, with a suspension of a two-month-old culture grown on 5% glycerin agar. Inoculations totalling 2 cc. were made on the forehead, cheeks, breast and left groin. Four days later a large nodule appeared on the left cheek where the largest dose had been given, and within a few days all three nodules

on the face were exuberant. On February 5 the nodule on the left cheek was biopsied; a smear proved very rich in acid-fast bacilli. After treating the triturated material with 10 per cent sodium hydroxide, the sediment obtained by centrifuging was sown onto 6 tubes of Loewenstein's medium. Six weeks later (March 22) five of the six tubes showed growths, similar to that of the original culture, which covered from one-half to three-fourths of the surface of the medium. Microscopic examination proved it to be pure and identical to the original strain. This was the first retroculture of the "Dalva" strain.

RESÚMEN

El autor logró cultivar, de las secreciones nasales de un paciente leproso, un bacilo ácido-resistente virulento en animales experimentales. El cultivo, llamado cepa "Dalva," es no-cromogénico y de tipo R. Es positivo a la prueba Dubos, tanto como el bacilo de Koch, pero no produce lesiones tuberculosas en cobayos, al contrario produce lesiones focales purulentas en varios órganos rodeadas de células mononucleares y con abundantes bacilos. También se produjeron lesiones en un mono (*cebus fattuellus*).

El autor sugiere que se trate de obtener nuevas cepas de bacilos de las secreciones nasales de pacientes lepromatosos.

DESCRIPTION OF PLATE

PLATE 17.

FIG. 1. The nonchromogenic "Dalva" culture, obtained from the nasal mucus, on Loewenstein's medium. The first tube (#33), the original culture, was nearly six months old when the photograph was made (June 3, 1951). The other two tubes (#34) show the appearance of the second generation, transplanted on February 17, 1951, after somewhat more than 2½ months of growth.

FIG. 2. Smear of the "Dalva" strain stained by Fontes method. All bacilli appear granulated.

FIG. 3. Section of the subcutaneous abscess produced by the "Dalva" bacillus in the guinea-pig. $\times 64$.

FIG. 4. Section of the same lesion, in the abscess area, showing a large mass of bacilli stained by the Ziehl-Neelsen method, and also scattered intra- and extracellular bacilli. $\times 500$.

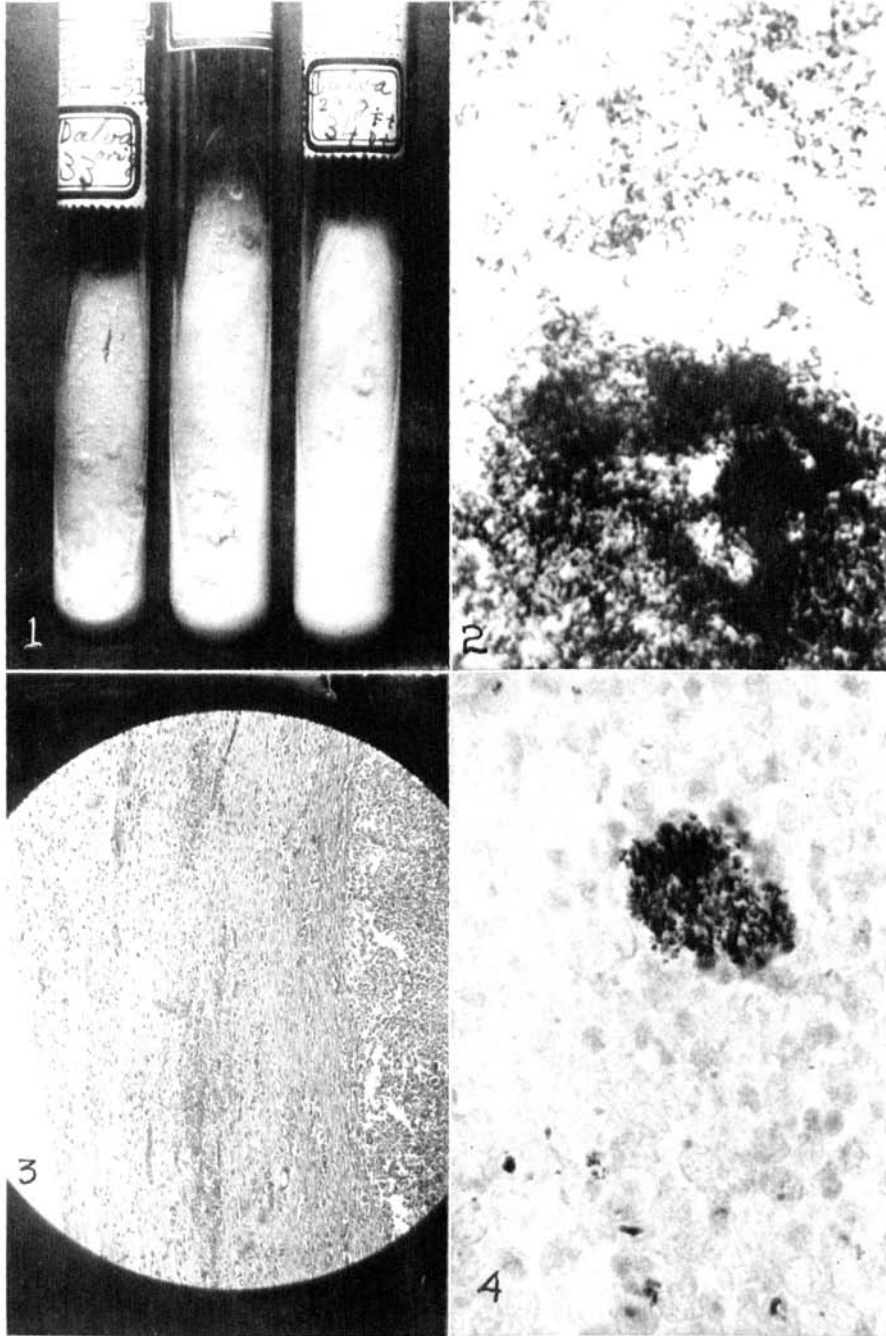


PLATE 17.