

bacilli with hot alkali. Flocculation did occur with the extracts prepared according to Castaneda's methods.

In a smaller series of tests, White<sup>2</sup> reported essentially the same results. He, therefore, feels that the somatic complex of *B. proteus X19* presents 2 distinct serological factors: one labile in hot alkali and responsible for O agglutination in its own antiserum without having any part in the Weil-Felix reaction; and another stable in hot alkali and responsible for the Weil-Felix reaction.

As to the chemical nature of the reacting substance, Castaneda and White report that this substance gives a powerful Molisch reaction, withstands boiling in alcohol or water and a negative biuret reaction. The extracts we prepared from the 0-504 strain of *B. proteus X19* reacted in the same way.

In using the alkali extract, we observed that it is necessary to adjust the H ion concentration within the limits of pH 7.2 and pH 7.4. Extracts which are too acid or too alkaline are liable to spontaneous flocculation at the incubation temperatures we employed.

We had difficulty in controlling the potency of hot alkali extracts which, although subjected to the same treatment, did not yield as much reactive substance as did similar preparations. We are unable to offer any explanation for this. One-day cultures do not yield as much reactive substance as 3 to 4-day cultures at 37°C.

Our work does not permit us to draw conclusions as to time in the disease when the reacting antibody may appear in the patient's serum. For this study we purposely selected sera which had a high reactive quality as indicated by their agglutinating titer. From the few tests we made with serum taken shortly after the onset of the disease, it would appear that significant flocculating titer parallels that of agglutination.

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#### Morphological and Tinctorial Behavior of *B. Leprae* During its Adaptation to an In Vitro Habitat.

CHARLES W. DUVAL.

*From the Laboratory of Bacteriology, Tulane University Medical School.*

Much has been written concerning the variations in morphology and amphoteric staining property with respect to acid-fastness for the supposedly cultivated Hansen bacillus of leprosy during that

period in which the organism is acquiring the power to grow saprophytically. Confusion and doubt over the authenticity of the *in vitro* culture have arisen, because of the curious results obtained by various workers. These results presuppose that the specific microbe of human leprosy is of such protean pleomorphism that it may alternately appear as a non-acid-fast diphtheroid, as both acid-fast and non-acid-fast streptothrix, as a chromogenic and non-chromogenic acid-fast bacillus.

Kedrowski,<sup>1</sup> Deycke,<sup>2</sup> Bayon,<sup>3</sup> *et al.*, believe that *B. leprae* is capable of extreme pleomorphism and change in tinctorial properties which explains the non-acid-fast streptothrichal and filamentous "stages" reported by them for the initial cultivation. Recently, Denney<sup>4</sup> reports what he regards as biological evidence that the "globi" in the human leprosy lesion may be analogous to the sporangial or zooglic stage of certain higher bacteria or protozoa. Salle<sup>5</sup> claims that *B. leprae* in culture upon living tissue (Carrel's medium) multiplies very slowly and appears both as acid-fast and non-acid-fast diphtheroidal forms. However, when the culture is subplanted to ordinary nutrient media a luxuriant "orange" colored growth occurs, the individual bacilli of which are now non-acid-fast.

Thus the literature reports a bewildering number of "stages" and transformations for *B. leprae*, which do not conform with our conception of metamorphosis for a single microorganism. However, the Hansen bacillus of leprosy, like many other parasitic microorganismal species, may exhibit minor changes in morphology while adapting itself to a saprophytic existence.

To determine the true morphological and tinctorial behavior of *B. leprae* during its adaptation to live and multiply in an artificial medium, the subcutaneous leprosy nodule from 4 cases of human leprosy was secured for study through the kindness of Dr. Denney, physician in charge of the National Leprosarium at Carville, Louisiana. Each nodule was rich in the acid-fast-rods of Hansen and free from any cultivatable contaminant.

The leprosy material was prepared and handled in the following way: (1) parts of each nodule were fixed in Zenker's fluid, paraffin embedded, sectioned and stained for acid-fast organisms; (2) emulsified bits of leprosy were first treated with 1% solution of

<sup>1</sup> Kedrowski, *Z. f. Hyg. u. Infektionskr.*, 1901, **37**, 52.

<sup>2</sup> Deycke, *Ges. Dtschr. Naturf. u. Arzte*, 1910.

<sup>3</sup> Bayon, *Scientific Memoirs, Government of India*, 1911, **42**, 1.

<sup>4</sup> Denney, *U. S. Public Health Reports*, 1934.

<sup>5</sup> Salle, *J. Infect. Dis.*, 1934, **54**, 347.

NaOH for one hour, then washed in sterile Ringer's solution before planting upon culture media; (3) small pieces of leprous tissue were submerged in 1% sterile trypsin solution for 24 hours at 37°C., after which the softened material was transferred to a variety of culture media; (4) smear preparations of the juice from macerated bits of the untreated fresh leprous tissue were stained by Ziehl-Neelsen's, Loeffler's, Gram's and Gabbet's methods, respectively; (5) bits of untreated fresh leprous tissue were planted upon a variety of nutrients including living chick embryo and growing tissue *in vitro*. Microscopic studies of the various materials were made every 3-5 days, over a period of approximately 6 months, which, in our hands, was the time required by *B. leprae* to become established in culture as a macroscopic growth.

*Morphological features.* The multiplying *B. leprae* of the initial culture shows only minor alterations in size, shape and arrangement of the chromatin from that observed for the bacillus in the human lesion. The leprosy "globi" which the author regards as intracellular colonizations, do not occur in culture even when the nutrient employed is living tissue. Such changes, however, as do occur are not as marked as those seen for the tubercle and diphtheria bacilli when cultured away from their natural habitat. In no sense are the morphological variations to be considered in the category of developmental "stages" in the life cycle of the organism. Furthermore, the slight pleomorphism of the Hansen organism is only noticeable during its period of adaptation to a purely saprophytic environment.

Under cultivation the leprosy bacillus early loses its sharply circumscribed dense bacillary masses known as "globi". In cultures that are established, these curious formations are never seen. The "globi" in tissue transplants become larger in size from week to week, due apparently to the increase in number of their contained bacilli. Later the "globi" lose their sharpness of outline when a separation and spread of the bacilli occurs into the surrounding medium. These changes to the "globi" are regarded as the forerunner or early sign of *in vitro* multiplication.

With regard to the individual bacilli, many become distinctly larger, less curved, more plump and rounded instead of pointed at the ends. Changes in the distribution of the chromatin also occur in that the so-called "beads" or "granules" in the bacilli become larger and more numerous. Certain rods have a sharply defined granule at either pole, while others possess only a large centrally located granule, and still others may have 4 to 6 granules. The bipolar arrangement of the granules accounts for the leprosy organ-

ism in stained preparation resembling certain forms of the diphtheria bacillus from culture. *B. lepræ* at this period of its growth is distinctly diphtheroidal. In culture, the viable bacilli are always outlined by a cell-wall or membrane.

Before there is any definite evidence of macroscopic growth the transplanted bits of leprosy tissue contain great numbers of extracellular granules which are non-acid-fast though positive to Gram's method of staining. These extracellular granules are chromatin masses and are similar to the "Much" granules in the lesion of tuberculosis. While some writers regard them as "degeneration" forms, others believe that they may be analogous to certain spores. Germination of these "bodies" has not been observed, though carefully studied with this point in mind. The fact that these extracellular granules appear in great numbers in early cultures during the period of saprophytic adaptation supports the view that they do represent some sort of transitional stage of the organism. It is possible that these granules are the forerunner of the permanent bacillary forms that have adapted themselves to the new environment. In this connection it is noteworthy that when the leprosy bacillus has completely acquired the power to grow saprophytically, these "free" granules are not observed.

About the time the leprosy culture becomes visible to the naked eye, the bacilli appear plump, short and ovoid. Intracellular granules are conspicuous by their absence, because the chromatin is now homogeneously distributed. During the transitional period the bacilli show a well defined cell membrane. Still later, however, when the culture will grow upon ordinary laboratory media, the bacilli for the most part become again distinctly "beaded" with metachromatic granules.

*Tinctorial Properties.* The viable Hansen bacillus of leprosy whether *in vivo* or *in vitro* is always acid-fast in the generally accepted sense of the term. Its resistance to certain strengths of decolorizing agents is as constant as that of the tubercle bacillus; however, it will not resist decolorization to the same degree as will the latter. The Ziehl-Neelsen and other methods used for staining tubercle bacilli in tissue, will not satisfactorily demonstrate the leprosy organism under the same conditions. Likewise, these methods are unsuitable for discernment of *B. lepræ* from culture, unless a weaker strength of the acid-alcohol decolorizer, or a shorter period of treatment is employed.

At no time during the period (6 months) in which *B. lepræ* is adjusting itself to artificial growth conditions, was there noted

any difference in respect to its acid-fastness. Throughout the entire 6 months' period of microscopic growth, the same degree of resistance to decolorizing agents was observed for the bacilli in the transplanted leprous tissue as is the case of the organism in the human leproma. There is, however, far less resistance to decolorization during the transitional stage that just precedes the initial macroscopic growth. In the opinion of the author this fact may explain the statement of Salle that the subculture of *B. leprae* from Carrel's living tissue medium to ordinary nutrients, becomes non-acid-fast.

*B. leprae* is at all times strongly positive to Gram's method of staining. Where this method is employed without counter-staining, the individual bacilli simulate short chains of streptococci. This appearance is due apparently to the retention of the stain by the denser chromatin masses. Gram's method, more than any other, accentuates in "bas-relief" the chromatin granules. Differences in behavior of the leprosy bacilli to the alcohol treatment in the Gram's method have not been observed. In other words, the so-called involutions, transformations, degenerations and chromatin granules are equally positive in their reaction to this method of staining.

*B. leprae* when stained by Loeffler's methylene blue method for *B. diphtheriae* reveals the fact that the intra- and extracellular granules are distinctly metachromatic. Many of the stained bacilli from early cultures are striking in their morphological appearance to the bipolar forms of *B. diphtheriae*. Undoubtedly this would account for the diphtheroidal "stage" of the leprosy organism referred to by some writers.

*Summary.* The uncontaminated subcutaneous nodules from 4 cases of human leprosy were cultured upon a variety of media and under various conditions, in order to afford a systematic study of the morphology and tinctorial properties of *B. leprae* during its period of adaptation to an artificial environment. All changes occurring for the Hansen bacillus *in vitro* were noted and compared with the morphology and staining properties of the organism in the original human leprous lesion.

During the entire period of observation (6 months) only minor alterations in size and shape of *B. leprae* were noted, and these were no more than what occurs for the tubercle bacillus under similar conditions of cultivation. Where multiplication occurred in the transplanted leprous tissue the acid-fast rods became longer, thicker, less curved and more distinctly diphtheroidal in appearance,

because of the bipolar massing of the chromatin. So striking was this arrangement of the chromatin granules that with Gram's and Loeffler's methods of staining, certain of the *in vitro* growing forms are indistinguishable from the bipolar metachromatic granular types of *B. diphtheriae*. Later, when the organism is acquiring the power to grow independently of the host tissue it becomes shorter, more plump and often without granules. Still later, after macroscopic growth is well established, the bacilli assume the morphology of those in the transplanted leprous tissue. At no time were branching filamentous and interlacing streptothrichal forms encountered.

The carbol fuchsin stained microorganism of leprosy, whether in culture or the living host tissue, is less resistant to decolorization by the ordinarily used agents for this purpose, than is the tubercle bacillus. Failure to realize this fact may account for the reports of non-acid-fast diphtheroidal and streptothrichal forms of the Hansen organism. *B. leprae* in the human lesion is decidedly more resistant to decolorization than it is from macroscopic culture. Where the Ziehl-Neelsen method is employed there occur varying degrees of acid-fastness to complete decolorization of the bacilli from culture. In a given field of the stained preparation there will be rods that have only partially, and still others that have not, retained the stain. On the other hand, where the Ziehl-Neelsen method is used and the time-period for decolorization is greatly lessened, the leprosy bacilli will remain deeply stained.

The Hansen organism of human leprosy is an acid-fast, Gram-positive bacterium that possesses much the same morphology and staining properties as the tubercle bacillus.

Finally, in the experience of the author,<sup>6</sup> the macroscopic culture of *B. leprae* is obtained only after months of careful cultivation upon split-protein media, preferably that of the excised, transplanted autolyzing leproma. Once the organism has successfully passed through the transitional period of *in vitro* adaptation, growth takes place readily upon the ordinary laboratory nutrients.

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<sup>6</sup> Duval, C. W., and Holt, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1934, **31**, 828.