6578

Non Acid-Fast Rods and Granules in Vertical Sections of Mycobacterium Tuberculosis Colonies.

MORTON C. KAHN AND JOSÉ F. NONIDEZ.

From the Departments of Public Health and Anatomy, Cornell University Medical College.

The results of an investigation on the development process of the human *Mycobacterium tuberculosis,* using the single cell method of approach, were reported.[1] Single organisms or small groups of from 2 to 6 were isolated in micro-droplets of Long's medium. These micro-droplets were preserved in such a manner that periodic observations could be made on identical individuals for several days or weeks. While variation did occur, the most usual type of reproductive process was the segmenting of the rod into 3 or more ovoid units, the reduction of these units into fine granules from which, later, extremely fine and delicate rods were found to develop. These rods elongated and thickened with varying rapidity until they became of the size and shape of what may be called the mature acid-fast tubercle bacillus. In the fine granule phase of the development process, as well as in the case of the exceedingly small and delicate rods which were observed to evolve from the granules, the organisms appear distinctly non-acid-fast.

The changes which were described are seemingly not allied to those recently described by Mellon and his coworkers[2] for the avian *M. tuberculosis,* in which a filterable phase is involved. In a recent study of one of us (K) no positive results with human *M. tuberculosis,* as far as filterability is concerned, have been obtained by culture or animal inoculation when the N, V, or W Berkefeld candles are employed, or with the neutrally charged membrane filters of Zsigmondy-Bachman. An occasional filtrate was found to become clouded with coccoid and diplococcoid types after long incubation, but upon critical examination the organism which grew up was found to be a non-pathogenic member of the diphtheroid group, which at times produces a considerable amount of acid-fast material and therefore could possibly be mistaken for *Mycobacterium tuberculosis.* An organism having the identical characteristics of this diphtheroid has purposely been isolated from the air. Another point

[1] Kahn, *Am. Rev. Tuberculosis,* 1929, **20,** 150.

[2] Mellon, Richardson and Fisher, *J. Bact.,* 1933, **25,** 45.

of departure from the work reported by Mellon and his coworkers lies in the fact that the morphological changes which we described took place in the identical microdroplet of medium in which the original single cell was isolated. Transfers to different types of media were not found necessary, and accordingly any possibility of contamination from the air was avoided. Nor was it possible to fix the organism in any one of the morphological stages. Changes, when they did occur, were invariably found to be progressive in nature.

Oerskov[3] and Bergel[4] have considered the non-acid-fast rods and granules to be degenerative forms of the *M. tuberculosis*, appearing in greatest number in old cultures. One of us (K) has not been able to substantiate this claim upon examining cultures of from 10 days to 6 months of age. It is true, however, that the non-acid-fast rods and granules are not readily recognized by those unfamiliar with the above mentioned development process, when the usual Ziehl-Neelsen staining method is applied to direct smears obtained from culture on liquid or solid medium. The following technic has been successfully applied by one of us (N) to the making of vertical sections of colonies grown on egg medium and also to growth membrane developed upon the liquid medium of Long. Upon examination these sections revealed large numbers of unmistakably non-acid-fast rods and granules which, as will be described, occur in such a position as to strongly suggest their being the youngest forms of *M. tuberculosis*.

Small blocks of the medium with the attached colonies were fixed in 95% alcohol for 24 hours, dehydrated with absolute alcohol and cleared with xylol. They were embedded in paraffin and cut into vertical sections 4 micra in thickness. The sections were fixed to the slide with a minimum amount of albumen glycerin mixture. The paraffin was dissolved with xylol, the sections rinsed once more with pure xylol and flooded with absolute alcohol to remove any trace of the paraffin solvent. They were stained with the Ziehl-Neelsen method and after drying were covered with a drop of damar and a cover glass added. The same procedure was followed for the study of the growth membrane taken from liquid medium, but the latter was found to be crumbly and had to be handled very carefully to prevent its disintegration.

In order to meet possible objections to the action of such fat solvents as alcohol and xylol on the organisms, the procedure used

[3] Oerskov, *Z. fur Bakt. Parasit. u. Infekt.*, 1932, **123**, 271.

[4] Bergel, *Z. Tbk.*, 1914, **22**, 343.

for the preservation of fats and lipoids was also applied. Colonies attached to the egg medium were fixed in 15% neutral formalin for 48 hours. They were then suspended in a 3% solution of warm agar. After cooling, a block was trimmed. The block of agar enclosing the colony was placed upon a drop of distilled water on the stage of the freezing microtome and frozen with liquid CO_2. Vertical sections of the colony were cut. The sections were lifted from the edge of the knife with a fine brush and put into sterile distilled water, where they spread. They were transferred to slides smeared with albumen fixative, spread flat, allowed to dry, and stained with the Ziehl-Neelsen method in the usual way, finally being mounted in damar under a cover glass. The about to be described non-acid-fast rods and granules appeared in both the colonies treated with alcohol and xylol, and in the sections of colonies cut with the freezing microtome on which no fat solvent was used. The presence of these non-acid-fast forms cannot, therefore, be attributed to the uneven dissolution of the fatty substance by the reagents mentioned above. If alcohol and xylol do exercise any influence on the staining capacity of *M. tuberculosis,* the latter would seldom have been seen in its acid-fast condition in sections of tuberculous tissue when prepared with the ordinary histo-pathological methods, for the organisms occur in small numbers and usually scattered. Unless the penetration by xylol be thorough, it would be impossible to cut sections of such material. As a further control, however, a number of thin smears were made from a growing colony of *M. tuberculosis.* Some of the slides were stained with the Ziehl-Neelsen method as controls, while the others were placed in a mixture of equal parts of absolute alcohol and xylol for 24 hours. They were thoroughly rinsed with absolute alcohol, allowed to dry, and then stained with the aforementioned technic. No relative increase of non-acid-fast to acid-fast organisms occurred. In other words, though the mycobacteria were thinly scattered over the slide, 24 hours in these reagents did not influence their subsequent staining. A similar experiment was performed with tuberculous sputum some years ago by Dr. Ralph Stillman of this institution, also with negative results.[5]

Finally, a word must be said for the use of albumen for fixing sections to the slide. Albumen fixative is a mixture of equal parts of the whites of fresh eggs and glycerin. It is carefully filtered after preparation. When spread thin upon a clean slide, it does not take the stains, hence its current use in histology. In order to make sure that no background could result from the use of this mixture in any

[5] Personal communication.

way simulating micro-organisms, and that the paraffin used for embedding did not leave a confusing residue, paraffin sections not containing the colony were fixed to slides smeared with the albumen glycerin.* After the paraffin was dissolved with xylol and the slide rinsed with absolute alcohol, the staining method of Ziehl-Neelsen was applied. Nothing resembling rods or even granules could be seen in these preparations, the background being entirely colorless.

Upon examination of a vertical section of a human or bovine *M. tuberculosis* colony some 6 weeks of age, which has been stained with the Ziehl-Neelsen method, the low power of the microscope will reveal 3 distinctly stratified zones. The lower zone, in closest contact with the medium at its base, extends up through the colony some two-thirds to four-fifths of its entire depth and appears strongly acid-fast. The remaining peripheral zone may be divided into 2 parts, the outermost of which appears definitely non-acid-fast. Directly under this there is a very narrow but distinct area which appears as a stratum of indefinite pale red color, considerably less strongly acid-fast than the lower area above mentioned. When an examination is made with the oil immersion magnification, the outer peripheral zone is found to be composed of myriads of non-acid-fast rods and granules, as well as acid-fast rods containing the dark appearing intracellular areas. The lower stratum, which is strongly acid-fast, is composed principally of large, strongly acid-fast rods, with a few non-acid-fast rods and granules scattered through the cracks and crevices which appear in this portion of the colony. The lighter staining central stratum is composed principally of pale acid-fast rods and granules, although relatively a considerable number of non-acid-fast forms are also encountered in this locality.

The question at once suggests itself as to which is the older portion of the colony, the peripheral zone or the lowest stratum. If the peripheral zone were the oldest, then the claims of Oerskov—who believes the non-acid-fast rods and granules to be old degenerative forms—may well be correct. We, however, believe that the reverse is true, and that the peripheral zone containing the myriads of non-acid-fast rods and granules is the youngest portion of the colony and the zone of active growth. Our reasons are: 1. When vertical sections are made through old colonies of *M. tuberculosis* which have ceased their growth, the peripheral zone is almost entirely obliterated, there being only a few non-acid-fast forms scattered here and there at the extreme outer edge of the colony, while the remaining areas appear strongly acid-fast. 2. Vertical sec-

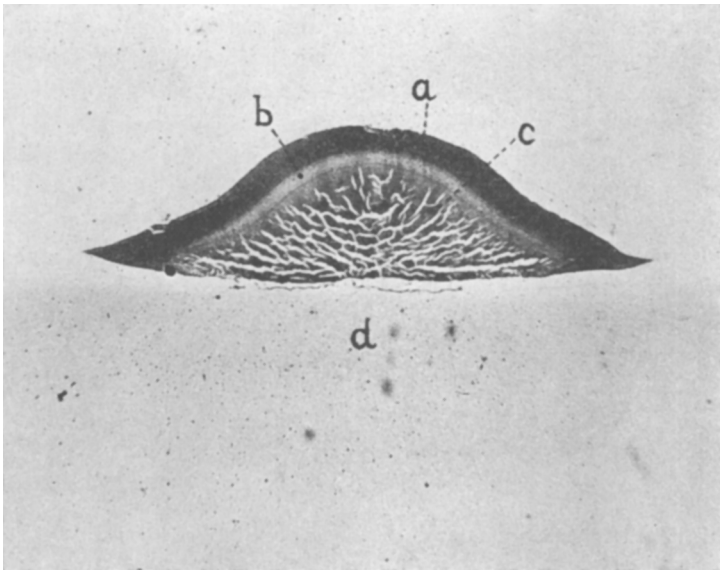

FIG. 1.

PHOTO-MICROGRAPH OF STAINED VERTICAL SECTION OF MYCOBACTERIUM TUBERCULOSIS COLONY. ×200.

The peripheral zone A contains myriads of non-acid-fast rods and non-acid-fast granules, as well as young acid-fast rods which contain the dark staining intracellular elements. Zone B contains the lighter staining but definitely acid-fast rods and granules. Zone C is composed chiefly of large, strongly acid-fast rods, with a few non-acid-fast rods and granules occurring in the cracks and crevices. Zone D, egg medium. The space between the colony and the egg medium is of course due to shrinkage. The cracking of the acid-fast core of the colony in this case is an artefact due to the drying of the sections prior to Ziehl-Neelsen staining. However, when silver nitrate is used for impregnation and the sections are not allowed to dry, a number of well defined vacuoles are present in this strongly acid-fast "medulla", which give additional evidence that this portion of the colony is the oldest, for these vacuoles do not appear in the peripheral zone.

tions have also been made through young and old growth membrane taken from Long's liquid medium. The young, thin, white growth membrane reveals myriads of non-acid-fast rods and granules, with the beaded acid-fast forms scattered here and there through the mass, while the yellow, crusty, folded, older area of growth membrane gives rise to relatively few of the non-acid-fast types. 3. The mycobacteria at the periphery of the colony are presumably receiving the most oxygen and the exact oxygen requirements of this organism are well known. 4. The single cell studies on the development process reported by one of us (K) indicated the non-acid-fast rods and granules to be the youngest form of *M. tuberculosis,* and also that the acid-fast rods with the dark intracellular elements were

the first step in the formation of a new generation. The information obtained from a study of the vertical sections of both human and bovine colonies of *M. tuberculosis* would seem to substantiate this claim.

It seems as though we must conceive of the colony as a half sphere and presumably some growth is taking place throughout the entire area as evidenced by the few non-acid-fast rods and granules which may be seen scattered even through the strongly acid-fast "medulla". The peripheral zone, however, reveals considerably larger numbers of these forms, and is therefore presumably the area in which most active growth is taking place.

6579

Precipitins Against Fractions of Streptococci in Hemolytic Streptococcus Disease, Glomerular Nephritis, Rheumatoid Arthritis, and S. Viridans Endocarditis.

DAVID SEEGAL, MICHAEL HEIDELBERGER, ELIZABETH L. JOST AND JOHN D. LYTTLE.

From the Department of Medicine, College of Physicians and Surgeons, Columbia University, the Presbyterian Hospital, and the Department of Pediatrics, Babies Hospital, New York City.

The sera of 310 patients have been studied for precipitins against 2 protein fractions of *S. hemolyticus*, the nucleoprotein of *S. viridans* and the group specific carbohydrate of *S. hemolyticus*. The *S. hemolyticus* fractions were freshly prepared. The *S. viridans* protein first used* was 5 years old. Later tests with a sample newly isolated from the same strain gave identical results. A description of the precipitin test and the antigens used has been presented in a previous communication.[1]

Sera from the following groups of cases have been studied:

1. A control group of 39 healthy nurses during the fall season.†
2. A control group of 16 healthy medical students and nurses during the spring season.

* Kindly supplied by Dr. Rebecca C. Lancefield of the Hospital of the Rockefeller Institute for Medical Research.

[1] Seegal, D., Heidelberger, M., and Jost, E. L., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 939.

† These data were made available by Dr. A. F. Coburn.