The Bacteriology of TUBERCULOSIS

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Preface

IN SPITE of great advances in the treatment and cure of tuberculosis, there are still millions of tuberculous people spread over the surface of the earth. Chemotherapy, the use of antibiotics, and surgery still leave resistant cases in which the disease continues to progress steadily. And while some problems in the bacteriology of tuberculosis have been solved, others – some previously unknown – have arisen. The survival of tubercle bacilli in organisms treated with antibiotics and chemotherapeutics, the relation of the cord factor to pathogenicity, the role of chromogenic bacilli in the pathogenesis of tuberculosis, the cultivation of tubercle bacilli from paucibacillary material – these are some of the new problems.

In the course of the past twenty years many works in the field of the bacteriology of tuberculosis have been published, dispersed in both well-known and less familiar periodicals all over the world. Before further advance in tuberculosis research can be made, it is necessary to summarize the results already achieved; this is one of the aims of this book. Another of my objectives is to point out unsolved problems in the bacteriology of tuberculosis and to indicate recently developed experimental methods for the study of tubercle bacilli.

Their limited knowledge of foreign languages and the pressure of time often make it difficult for the younger generation of scientists to trace the development of problems. Yet we really know a problem only when we are acquainted with its history. When scientists lack such historical background, apparently new contributions often turn out to be a repetition of work done previously. The avalanche of such repetitive work is rolling on and increasing in size. If it were possible to direct all this wasted energy upon previously untrod paths, the achievements would increase in value and the flood of publications would diminish. These considerations led me to present the material of this book in historical sequence, although through such an approach some well-known names will lose their priority as propounders of certain discoveries.

Preface

In my attempt to follow work in the bacteriology of tuberculosis up to this very day, I was forced to consider yet unfinished work. In such cases, as Claude Bernard has pointed out, it is only possible to gather the raw facts. These data will often be contradictory, but they may serve as centers for the generation of new ideas and work.

The number of persons, institutions, and editors who have in various ways been helpful to me in preparing this manuscript has, over the years, grown so great that it is impossible to mention all of them here; but I am deeply grateful for their friendly aid. The reader will find some of their names in the text itself. I would, however, like to express my particular gratitude to the personnel of the University of Minnesota Library for tireless assistance in collecting material for the manuscript and to the personnel of the University of Minnesota Press.

E. DARZINS

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PART ONE

Morphology and Cytology of the Tubercle Bacillus

I

Tubercle Bacillus as a Unicellular Organism

ROBERT KOCH (1882), in his paper on the discovery of the cause of tuberculosis, described the tubercle bacillus, stained with methylene blue and counterstained with vesuvin, in the following terms (p. 222): "The bacteria made visible through this procedure are somewhat peculiar in aspect. They have the shape of a rod and, because of this form, belong to the group of bacilli. They are very thin and one-fourth to one-half as long as the diameter of a red blood cell; sometimes they reach a greater length and are as long as the whole diameter of a red cell. Under certain conditions, the bacilli produce spores already in animal organism; some bacilli contain several spores, in most cases there are two to four of them; oval in form, they are distributed, in uniform intervals, along the axis of the bacilli."

The picture of the tubercle bacillus given by Koch was confirmed by the later and more efficient staining methods of Ehrlich and Ziehl-Neelsen. The tubercle bacillus belongs to the genus *Mycobacterium*. Three variants, *Mycobacterium tuberculosis* var. *hominis*, *bovis*, and *avium*, cause tuberculosis in mammals.

Early Upholders of the Unity of the Cellular World

Early investigators regarded the bacterial cell as a living unit endowed with the same constituents as the cells of higher plants and animals. These early upholders of the unity of the cellular world reached their conclusions by means of the incomplete data they got from the observation of living cells. Schottelius, as early as 1888, used reagents to determine the differences in chemical composition of the bacterial cell structures. He observed in the cells of *Bacillus anthracis* a dark central body in the transparent protoplasm which he called a "nucleus" to designate its central position. He affirmed that the cell division of these bacilli is preceded by the division of the central body, and expressed the hope that further investigations

Figure 1. Unstained tubercle bacilli; oblique illumination; × 1,000 (Lembke and H. Ruska, 1940).

would furnish additional information about the nature of the central body.

Two works, by Ernst (1889) and Babes (1889), advanced the knowledge of the internal structure of the bacterial cell to the point that there was practically no progress in later works till new tools of investigation were created. The significance of the work of Ernst and Babes in bacterial cytology has been overlooked by the latest reviewers of this field.

Ernst demonstrated, by means of simple procedures, the nuclear nature of granules of the bacterial cell. He showed that these granules are not vacuoles, nor are they of fatty nature because they are not dissolved by fat solvents. The cell granules, like any other nuclear substance, were stained in deep violet by hematoxylin. By treating these cell granules with pepsin and hydrochloric acid, Ernst showed that the granules are less resistant to enzyme digestion than are bacterial spores. He observed that the granules take part in the formation of spores and that the division of the granules takes place prior to cell division. The sum of these observations enabled Ernst to conclude that the granules in the bacterial cells are of nuclear nature.

Besides nuclear bodies, fat and glycogen granules were discovered in the bacterial cell. Grimme (1902) found that a large number of the granules of bacterial cells were dissolved in hot water or by diluted acids and that they disappeared under unfavorable conditions of nutrition and reappeared when the conditions improved. Grimme named such granules "volutin" and regarded them as the stored food of the cell. This view was accepted by Fisher in 1903 and by Guilliermond (1910).

The clear concept of the first unitarians, Ernst and Babes, who regarded the bacterial cell, like the cells of higher living forms, as endowed with the nuclear substance but living independently, was obscured by the mosaic of observations that followed.

The works and discussions of later investigators mostly took the form of a controversy about the presence of volutin in bacterial cells and es-

pecially in tubercle bacilli. The presence of volutin in tubercle bacilli was affirmed by Babes (1910), Guilliermond (1910), and Hollande and Crémieux (1928), but denied by Kirchensteins (1922), Lewis (1941), and others.

Some investigators described the nucleus of the bacterial cell as a separate body which can be differentiated from the cytoplasm, fat, and volutin (A. Meyer, 1912). For others, the nucleus in the bacterial cell was made up of chromidial granules dispersed in the cytoplasm (Guilliermond, 1933), or the bacterial cell was devoid of any structures at all. According to this last view, the morphologic elements seen in the cells were created by the action of external agents upon the colloids of the cytoplasm (Wámoscher, 1930).

Opponents of the Theory of the Unity of the Cellular World

The hypothesis that the cell of the tubercle bacillus is composed of living units endowed with properties different from those of the whole bacillus emerged from the discussions of the nature of the formations seen in the tubercle bacillus by Koch. He identified these as spores, although it was early recognized that these bodies are not comparable to the spores of ordinary bacilli. The sensitivity to heat of the tubercle bacillus was alone sufficient to refute such an assumption. For some authors, the bodies were simple granules (Metschnikoff, 1888); others identified them as fat droplets (Grimme, 1902), volutin (A. Meyer, 1912), or as nuclear substances (Feinberg, 1900; Guilliermond, 1908; Eisenberg, 1909; Kirchensteins, 1921, 1922).

Spengler (1905a) approached the problem of the role of the bodies in the tubercle bacilli from a new angle. According to him, the tubercle bacillus is composed of living units, the *Splitter*. Their size is in the limits of microscope resolution, and they are acid-resistant but cannot be stained by the Ziehl-Neelsen technique. These *Splitter* are capable of reproducing new bacilli and may cause tuberculosis.

Much (1907 a, b), by applying to the tubercle bacilli Gram staining with successive iodine treatment and decolorization of the preparation with an alcohol-acetone mixture, was able to reproduce granules in the inside of the bacilli as well as scattered around them. These granules, according to Much, cannot be stained by the Ziehl-Neelsen technique but may generate new tubercle bacilli.

The work of the Brazilian investigator Fontes stimulated new interest in the cell structure of tubercle bacilli. In his first publication (1909), Fontes related how he had applied double staining to the bacilli, namely Ziehl-Neelsen's carbolfuchsin staining and the Gram treatment. In this way he tried to differentiate the pathogenic tubercle bacilli, containing Much granules, from the apathogenic ones without these granules. In his second

paper (1910a), Fontes described the multiplication through division of these granules in the inside of a cell and on its outside and applied the term "virus" to these formations. In another paper (1910b), Fontes described the application to the tubercle bacillus of the well-known method of separating the virus from the substrate by filtering the material through a bacterial filter. He inoculated a guinea pig with the filtered caseous material and transplanted the organs of this animal into a fresh one. When after five months of observation the animal was killed, the autopsy revealed the infiltration of round cells, granules, and occasional acid-fast bacilli in the lymph nodes and the lungs.

After years of oblivion, the early works of Fontes were rediscovered by Vaudremer (1923). He repeated Fontes' filtration experiments and confirmed the development of acid-fast bacilli on media and in animals inoculated with these filtrates. Vaudremer's article provoked a great quantity of research. The filtrability of the tubercle bacillus or the tuberculous virus (the *ultravirus tuberculeux* of the French authors) and the new disease produced by it were described.

An attempt to deprive the tubercle bacillus of its position as a member of the well-separated group of mycobacteria and to connect it with non-acid-fast saprophytic microorganisms was made by Ferrán (1905). He described non-acid-fast members in the chain of evolution of acid-fast bacilli. These findings have been upheld by Kahn (1929), among others.

Gardner (1929), Oerskov (1932), Brieger and Fell (1946), and Roth (1949) reinvestigated these assertions but could not confirm them. The classical acid-fast form of the tubercle bacillus was not lost and non-acid-fast forms were not generated in the development process of tubercle bacilli. Wyckoff (1934) used micro-motion photography to study the reproduction of tubercle bacilli and found that the bacilli multiply by transverse division. This division continues for a long time after the growth in length of the bacilli has stopped. Because of this, old cultures contain an abundance of short rods. In the tubercle bacilli he found no evidence of any cyclic life phenomena.

After thirty years of strenuous efforts to disintegrate the cell of the tubercle bacillus into invisible fragments and to attribute to these parts the characteristics of a "tuberculosis virus" – characteristics not revealed by the whole bacillus – no tangible results have been achieved. The great amount of work dedicated to the problem of the filtrability of the tubercle bacillus has yielded very few scientific facts. The critically controlled experiments have not revealed any form of tuberculosis in which the disease was provoked by a virus or by non-acid-fast bacilli or in which the bacillus of Koch was not at work.*

* A complete account of the filtrability of tubercle bacilli and of the virus problem was given by L. Nègre in A. Calmette, *L'infection bacillaire et la tuberculose chez l'homme et chez les animaux* (Masson, Paris, 1936), pp. 93–122.

Tubercle Bacillus as a Unicellular Organism

The works of Spengler, Much, Fontes, and many others who regarded the tubercle bacillus as composed of living units, created great expectations and promised great contributions to the understanding and prevention of tuberculosis. As with time these expectations faded, the *Splitter* and granules became the object of purely academic interest.

A new era dawned when the exact methods of physics and chemistry were applied to research on the cell. The investigation of bacterial cells in ultraviolet light, with an electron microscope, and by means of the histochemical and enzyme methods produced a considerable advance in the knowledge of the cytology of bacterial cells and particularly of the cytology of the tubercle bacillus. This type of investigation showed that the bodies in the bacterial cell are of nuclear nature and are connected with the assimilation, growth, and inheritance of the cell.

II

Physical Methods for Identifying Cell Structures

Ultraviolet Light Microscopy

THE resolving power of a microscope is inversely related to the wave length of the illuminant used; that is, the resolving power increases with decreasing wave length. For this reason, short-wave microscopy could be a considerable advance over earlier methods of penetrating into fine structures of the cellular world. But the practical realization of short-wave microscopy presented difficulties with respect to the source of the short-wave light and the material to be used for the lenses of the microscope and the slides. Light with a wave length of less than 300 mμ is ultraviolet, and ultraviolet light cannot penetrate glass. Köhler (1904) overcame these difficulties by using a homogenic linear light source of 275 mμ wave length which produced blue and violet monochromatic light from cadmium electrodes and by using a quartz objective and slides of mountain crystal. Finally, he attached a photographic device to this ultraviolet light microscope.

The investigation of the structure of the cell – particularly the bacterial cell – was the branch of biology that profited most from ultraviolet microscopy. Köhler himself took the first step in this direction. Living objects in particular attracted his attention. From photographs of the dust cells of the wings of a butterfly and the cells of a triton he concluded that the absorption phenomena of ultraviolet light in the structures of organic tissue are of special importance. (*"Ein hervorragendes Interesse bieten die Absorptionserscheinungen, die die organischen Gewebe diesem Licht gegenüber zeigen."*) The absorption of ultraviolet light was considerably greater in the nuclei than in the cytoplasm of the cells studied. Chromatin, the component of the nucleus, which in an ordinary light microscope is less transparent and which eagerly takes up the basic dyes, was also less transparent to ultraviolet light.

After the work of Köhler, important contributions in the field of the physical analysis of cell structures by means of ultraviolet light were made by Caspersson (1936, 1939, 1940, 1950) and his co-workers. The absorption of ultraviolet light by organic and inorganic compounds had been studied before Caspersson by Dhéré (1906). Dhéré's experiments showed that the radiations of wave lengths 274.8 mμ and 239.4 mμ are absorbed by a 1:10,000 solution of nucleic acid. It was known that the hydrolysis of nucleic acid produces phosphoric acid, carbohydrate, purine, and pyrimidine bases. Phosphoric acid and carbohydrate in the experiments of Dhéré did not absorb ultraviolet light, the absorption of ultraviolet light by nucleic acid being caused by its content of purine and pyrimidine bases. These investigations had shown the specific absorption maximum of nucleic acid to be in the region of 260 mμ wave length.

Caspersson developed the method of microscopic photometry, which permits the measurement, under a microscope, by means of a photoelectric cell, of the amount of different wave lengths transmitted by different parts of a cell. Two groups of substances in the cell showed strong absorption of ultraviolet light. The proteins, especially those containing cyclic amino acids, such as tryptophan and tyrosine, had this property, although the absorption of another group of substances, nucleic acids, dominated the phenomenon (see Fig. 2). The absorption of nucleic acids in the region of 260 mμ wave length was so high, and the absorption of other substances in comparison with it so low, that the absorption of these other substances can be disregarded. The conjugated double bonds in pyrimidine of desoxyribonucleic acid of the nuclei, caused this high absorption of ultraviolet

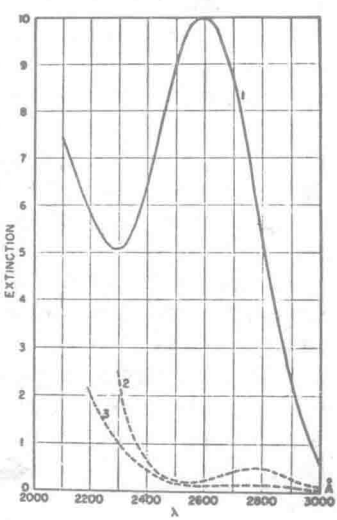

Figure 2. Comparison of absorption of ultraviolet light by nucleic acid and protein. 1. Thymonucleic acid, 0.5 per cent concentration. 2. Serum albumin, 0.5 per cent concentration. 3. Protamine sulfate, 5 per mill concentration. (Caspersson, 1950.)