CELLULAR STRUCTURES AND FUNCTIONS CONCERNED IN PARASITISM[1]

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The investigations carried out by Dr. Avery and his school between 1913 and 1940 have provided the pattern, the master plan, used by our generation for the immunochemical study of infectious processes. His more recent publications, on the other hand, have dealt with problems which bid fair to constitute some of the dominant preoccupations of the workers of tomorrow: namely, the nature of those modifications of host chemistry which result from infection, and the mechanism by which hereditary characters are transmitted in microörganisms.

The mere recital of his many distinguished and varied contributions would be sufficient to honor him. It would, moreover, give us the vicarious enjoyment of many historical discoveries: the correlation of the virulence of pneumococci with the possession of a capsule; the recognition that not only virulence, but also the immunological specificity of the different pneumococcus types depends upon the chemical characteristics of the capsular polysaccharides; the development of rational and precise concepts and techniques for the production of protective antibodies and enzymes selectively directed against the capsular material; the demonstration that many carbohydrates other than those of pneumococci can determine immunological specificity—for example those constituting the capsules of *Klebsiella pneumoniae* or, even more interestingly, simple sugars like glucose and galactose when incorporated into synthetic antigens. Careful study of his writings would reveal a subtle awareness of the complexity of the infectious process. The participation of the different components of the bacterial cell in virulence, immunity, and allergy—the multiple response of the host expressed not only by the classical immune and allergic manifestations, but also by the production of abnormal proteins of significance as yet unknown—all found their place in his analysis of the course of experimental and natural infections. Throughout his career we could enjoy with him many excursions into problems of bacterial physiology: the recognition of the exacting nutritional requirements of *Hemophilus influenzae* for the X (heme) and V (cozymase) growth factors; the striking oxidation processes and autolytic mechanisms of pneumococci; and last, but not least, the spectacular demonstration that a desoxyribonucleic acid fraction extracted from a culture of capsulated pneumococci can transfer to non-capsulated variants and their progeny the hereditary property to produce the capsular polysaccharide of the former culture, thus causing a directed hereditary alteration of the cell by means of a soluble, purified cellular component.

[1] Based on the Oswald T. Avery Lecture delivered before the Society of American Bacteriologists. Minneapolis, Minnesota, May 13, 1948.

All these achievements and their extension by other workers to many groups of microbial species constitute in a surprisingly large degree the subject matter and the doctrine of modern medical bacteriology. It is of interest, therefore, to strive for a better understanding of the manner in which Dr. Avery's work has altered the course of our science in the past and is now affecting many of our efforts and viewpoints.

Evolution of the concept of microbial parasitism. Although the concept of parasitism appears obvious in general biology, its meaning in relation to microbial infections remains very confused even today. This confusion results in part from the historical fact that our knowledge of microbial parasitism has evolved from two unrelated sets of phenomena, caused by entirely different mechanisms: on the one hand, the attack on plants and animals by visible predatory parasites; on the other hand, the processes of fermentation and putrefaction in their various forms. Although the relation of contagion to miasms, fomites and specific particles had been postulated many times before the bacteriological era, it is doubtful that any of the early students of disease had really reached the concept that microscopic living agents could attack man or animal. It is worth recalling in this respect that, even after he had become convinced that the pébrine corpuscles bore a causal relationship to the silkworm disease, Pasteur entertained for two years the view that the corpuscles were not truly independent living agents but were produced by the silkworm itself (34). It is almost certain that the discovery that certain infections, favus or scabies for example, were caused by fungi or by insects—parasites which could be detected with simple optical means—helped to breach the gap between the abstract concept of contagion and the concrete fact of parasitism. But whereas one could see or imagine how the arthropod attacked the host, it remained out of the range of experience to visualize how a bacterium or virus could possibly do it.

The relation of fermentation and putrefaction to disease processes had also been postulated for many centuries. Suffice it to recall here the oft quoted statement by Robert Boyle that "he, that thoroughly understands the nature of ferments and fermentations, shall probably be much better able than he, that ignores them, to give a fair account of divers phaenomena of several diseases, as well fevers as others, which will, perhaps, be never thoroughly understood, without an insight into the doctrine of fermentation".

The view that disease might be analogous to some derangement of orderly fermentation became more plausible when Pasteur traced the "maladies" of wine, beer and vinegar to the invasion of fermenting fluids by foreign germs capable of displacing or interfering with the microbes engaged in the legitimate business of manufacturing alcohol or acetic acid.

The participation of a foreign microscopic agent in obvious cases of parasitism (as in scabies) and in the spoiling of fermentation, appears today as sufficient justification for grouping under a general etiological concept many otherwise unrelated phenomena and to speak of contagious diseases as caused by parasites. But one may imagine that the nature of the relation between the chemical alterations during fermentation, the damage caused by a parasitic insect, and

the various infectious processes, must have appeared very obscure to many biologists. Indeed some must have wondered whether the similarity implied in using the word parasitism to discuss infectious diseases, went much beyond formal analogy and had any operational usefulness.

The biologically minded student of infection probably found comfort in the discovery by Metchnikov of the phenomenon of phagocytosis. As stated by Duclaux, there was at first sight something incongruous in a conflict between an ox and a bacillus; the sizes are so different (34). Since, according to Metchnikov, the bacillus was not dealing with the animal as a whole, but with phagocytic cells of the same order of dimension as itself, the relationship appeared more plausible and sensible. It is now known, in fact, that intracellular parasitism occurs in a great number of infections caused by bacteria and protozoa and that it is the rule in infections caused by viruses and rickettsia. In consequence, many aspects of microbial parasitism can be studied at the cellular level.

Unfortunately, transfer of the phenomena of parasitism from the level of the whole animal to that of the individual tissue cells did not make easier the analysis of the problem in mechanistic terms; the nature of the weapons used by the microbe to attack its host remained as obscure. Interestingly enough, progress in this direction has not come from classical descriptive biology, but rather from the observation of pathological processes and from attempts to unravel their chemical determinism. Recognition of the activities and nature of bacterial toxins was the first step in the analysis of the mechanistic aspects of parasitism. The evolution of our knowledge of these toxins began with the demonstration of the gross pathological effects of culture filtrates capable of reproducing many of the manifestations of the natural diseases; it was followed by the isolation in more or less pure form of some of the chemical substances responsible for these effects; it has now entered the phase of analysis of biochemical mechanism of toxin action, ushered in by the identification of the toxin of gas gangrene with the enzyme lecithinase, and by the brilliant hypothesis formulated by Dr. A. M. Pappenheimer, Jr. to correlate the action of diphtheria toxin with the inhibition of the cytochrome-b system. Other aspects of the evolution of our knowledge of bacterial toxins have just been reviewed by Dr. A. Bernheimer (14). These recent advances warrant the hope that it will soon be possible to account in biochemical terms for the toxemia which accompanies many bacterial infections, and for the other physiological and metabolic disturbances which constitute disease.

Granted the importance of these phenomena for the understanding of the clinical and pathological aspects of infection, they fail to reveal the primary cause of parasitism. Among microörganisms, very few can cause infection, and the problem of how the pathogens differ from their non-infectious relatives has perplexed bacteriologists since the beginning of our science. During the late 19th and early 20th centuries, countless studies were carried out in the hope of detecting metabolic or other biochemical differences correlated with infectiveness, but all in vain. Thus, it was discouraging to find that the ability of microorganisms to multiply *in vivo* is not correlated with any known nutritional and

other environmental requirements. In this respect, pathogenic bacteria apparently vary in a haphazard manner. Among acid-fast pathogens, for example, the leprosy bacillus has not yet been grown *in vitro*, the Johne's bacillus requires growth factors of unknown chemical nature, whereas the most virulent tubercle bacilli grow readily and abundantly in simple synthetic media. From another point of view, no valid evidence has yet been obtained that resistance to the bacteriostatic and bactericidal agents, or to the enzymes, normally present in tissue fluids or cells, determines the ability of a bacterium to survive and multiply in the body of a given animal. Neither is the ability to produce toxins a differentiating character. Typhoid or dysentery bacilli, for example, are not known to differ in this respect from many other gram negative bacilli which are only saprophytic; among group A streptococci, none is known to produce a more powerful erythrogenic toxin than the N. Y. 5, Type 12 strain which is essentially avirulent for all known experimental animals; or again, virulent and avirulent variants of tubercle bacilli are equally able to cause the toxic manifestations of tuberculin allergy.

Failure to explain infectiousness in terms of available biochemical knowledge led several workers during the first two decades of the present century to return to a more biological statement of the problems of infection. This attitude is well expressed in Theobald Smith's classical essay "Parasitism and Disease" (1934) in which infection is treated from the point of view of the ecologist.[2] In broad biological terms, the ideal parasitism was there conceived as a state of equilibrium between the infectious agent and the infected species permitting survival of both.

> "Parasitism is in a sense a compromise or truce between two living things, accompanied by predatory processes whenever opportunity is offered one or the other party. The universality of parasitism as an offshoot of the predatory habit negatives the position taken by man that it is a pathological phenomenon or a deviation from the normal processes of nature. The pathological manifestations are only incidents in a developing parasitism. As human beings intent on maintaining man's domination over nature we may regard parasitism as pathological insofar as it becomes a drain upon human resources. . . ."

Disease could then be considered as the multiple manifestations, conditioned by genetic and environmental factors, of this delicate equilibrium between invader and invaded host (76, 80).

This broad biological and ecological point of view has been extremely useful in the analysis of epidemiological problems, but it has contributed little to the understanding of the mechanistic aspects of parasitism. Nevertheless, some progress in this direction was made by analyzing host-parasite relationships in the general terms of the immunological reactions which are directed by the in-

[2] In the only long conversation which it was my privilege to have with Dr. Theobald Smith, he confided to me that his early studies on fermentation and other metabolic reactions had been motivated more by the hope of elucidating the nature of virulence, than by the necessity of working out diagnostic biochemical tests. It was after he had lost hope of reaching an understanding of virulence on the basis of existent chemical knowledge that he turned his attention to the broad biological laws of parasitism.

vaded host against the parasite as a whole. For example, one spoke of antityphoid, antiplague or anticholera antibodies to account for whatever resistance could be established against these infections. Furthermore, immunologists soon established that cultures of one given microörganism could elicit the production of several different antibodies; thus, a heat labile (H) and heat stable (O) antigen were recognized in several bacteria; antitoxic sera were differentiated from antibacterial sera; and the agglutinin absorption technique revealed that each bacterial cell constitutes an antigenic mosaic made up of several immunologically independent components. It was also suspected that certain bacterial constituents or products, loosely called aggressins, played a particularly important part in the phenomena of virulence. But despite this general awareness of the complexity of bacteria, the relation of the structure of the microbial agent to its ability to behave as a parasite had never been defined in precise terms. It is, I believe, the analysis of pneumococcus infections, carried out in Dr. Avery's laboratory, which provided the method by which antigenic structure, virulence, immunity and the other aspects of parasitism have been shown to be the expression of certain morphological and chemical characteristics of the microbial cells.

The mechanism of parasitism in pneumococcus infections. We shall not review the pioneer work of Neufeld and his school on specific pneumococcus immunity, of Toennissen on the carbohydrate nature of the capsule of Friedländer bacilli, and several other related studies, which constituted some of the material out of which Dr. Avery built the immunochemical laws which we are to consider now.[3] It is the bearing of these laws on the mechanism of microbial parasitism, and not the history of pneumococcus immunology, which is the object of our present discussion. The facts, and their relationships, can be outlined as follows.

a. There is a definite correlation between virulence of pneumococci and their possession of a capsule detectable by microscopic and immunochemical techniques. (4, 5).

b. The non-capsulated variants are more rapidly killed than the capsulated forms, both *in vivo* in the normal animal, and *in vitro* in mixtures of normal serum and leucocytes. The capsule participates in virulence by increasing the resistance of bacteria to phagocytosis. (4, 5).

c. The capsular substance is a polysaccharide. It varies in chemical structure from one pneumococcus type to another and conditions at the same time type specificity and virulence (4, 5, 46).

d. Specific antibodies directed against the capsular polysaccharides protect against infection by neutralizing the ability of the capsules to interfere with phagocytosis (4, 5); a similar result can be achieved with enzymes capable of hydrolyzing the capsular polysaccharides (6).

e. Although the reactive groupings of the capsular polysaccharides responsible for immunological specificity can survive many types of chemical and enzymatic treatment, the antigenic effectiveness of the complex polysaccharide antigen is

[3] A comprehensive survey of the historical aspects of this problem is given by B. White (86).

far less stable. In other words, the ability of capsulated pneumococci to elicit the production of antibodies protective against infection is optimum only when the bacteria used for immunization are prepared by techniques which maintain the antigenic integrity of the capsular structure (8, 11, 26, 27).

f. Immunization with pneumococci elicits the production of many antibodies directed against components of the bacterial cell other than the capsular substance (other polysaccharides, proteins, etc.) These other antibodies, however, have little if any ability to protect against infection although they may play a part in certain pathological processes, for example in those caused by allergic reactions to bacterial products (9, 47, 83, 84).

The significance of these facts can be emphasized from several different points of view. For many workers, the most important contribution made by Dr. Avery was the demonstration that carbohydrates play a part in virulence, antigenicity, immunity, and allergy equal to, if not greater than, the rôle classically attributed to proteins. Even more important, it seems to me, was the recognition that virulence and immunity can be analyzed apart from the parasitic cell as a whole, in terms of a few highly specialized components of it. In the pneumococci, these components were found to be the visible capsules, made up of polysaccharides; in other microbial species they might be other cellular structures, of another chemical nature. The important step, according to the view taken in the present discussion, was to have recognized the necessity, and the possibility, of analyzing infection not only in terms of general ecological host-parasite relationships, but in terms of identified cellular components of the parasite—its offensive and defensive weapons which effect the host and against which the host reacts.

This concept has now reigned over the study of infectious disease for a quarter of a century, and to enumerate its successes would be to recount the history of medical bacteriology during that period, an obviously impossible task. It is clear, however, that this approach does not constitute the only fruitful one to the study of infection; in fact, as was mentioned earlier, Dr. Avery himself has during the past ten years become vitally interested in other aspects of the problem of infectious disease, for example the altered chemistry and physiology of the infected host (1, 52), and the mechanisms of transmission of hereditary characters in microbial species (10, 57). Nevertheless, as the mechanisms which condition parasitism are the limited object of the present discussion, I shall attempt to discuss this problem further by applying the experience gained from the study of pneumococcus infections to the formulation of hypotheses concerning the pathogenesis of tuberculosis.

Tuberculosis as an example of parasitism. Tuberculosis illustrates well the problems of parasitism, because it presents a situation where the causative microörganism can live in apparent equilibrium with the infected host for prolonged periods of time, often causing only limited, or even no signs of clinical disease. The pathologist has described with extraordinary thoroughness the histological aspects of the response of the host to the parasite, from the initiation of the infection, to the establishment of the lesion, and its arrest or progression

to fatal outcome. The clinician has learned to recognize, predict and to some extent control, the manifestations of the disease. The epidemiologist has accumulated data describing the natural course of the disease in new and in immune populations, and the influence of environmental factors on morbidity and mortality. The bacteriologist has learned to grow the bacillus, and knows much of its peculiar chemistry. But despite this immense background of theoretical and practical knowledge of the disease and of the causative organism, little is known of the mechanisms by which tubercle bacilli become established in a new host, and cause disease, or of the processes used by the infected host to overcome the infection. In other words, we know much of the ecological aspects of host-parasite relationships in tuberculosis, hardly anything of the means used by the bacillus to behave as a parasite.

Of the many strains of acid fast bacilli which occur in nature, only very few can cause progressive disease in man or animals. The virulent forms exhibit a marked degree of specificity in their host range and have been classified on this basis into several pathogenic types: human, bovine, murine, avian, piscine. Nothing is known of the factors which determine specificity in host range.

Within any given pathogenic type, cultures of tubercle bacilli may vary greatly in virulence. This statement can be illustrated with a few specific examples of strains readily available to laboratory workers. The human strain H37 was isolated at the Trudeau sanatorium in 1905. In the course of time, two variants have been separated from it. One, H37Rv, is so virulent that a single or a very few cells can initiate progressive disease in guinea pigs (77) or susceptible mice (64); the other variant, H37Ra, is so devoid of virulence that even very large doses of it fail to cause progressive infection. Another strain, R_1, was also isolated from a patient at the Trudeau sanatorium; this strain however, soon lost much of its virulence and became stabilized at a level such that it fails to produce progressive disease in normal guinea pigs although it can infect silicotic animals (78). From this somewhat attenuated strain, there has also been isolated a variant (R_1Ra) which, like H37Ra, is so devoid of virulence that it cannot infect even silicotic animals. Other familiar examples of differences in virulence are the two bovine strains: Ravenel, which has remained highly virulent since it was first isolated some 50 years ago, and BCG which is sufficiently avirulent to permit its use as a living vaccine for immunization (19).

The relation of virulent and avirulent tubercle bacilli to phagocytic cells. Experimental pathologists have established many fundamental facts describing the comparative behavior of these different strains in resistant and susceptible animals. We shall consider for the present time only those facts which pertain to the behavior of the bacteria toward phagocytosis. Whereas, among pneumococci, the non capsulated organisms are much more readily phagocytized than the capsulated forms, no striking differences in rate of engulfment by phagocytes has as yet been described between virulent and avirulent variants of tubercle bacilli. Following their introduction into the animal (or in tissue cultures or other *in vitro* phagocytic systems) both the virulent and avirulent bacilli are rapidly taken up by polymorphonuclear leucocytes and find their way

into monomuclear cells a few hours later. It is the course of events subsequent to phagocytosis which differentiates the two forms of bacilli. The avirulent variants may survive intracellularly for prolonged periods of time, but fail to multiply to a significant degree. On the contrary, the virulent organisms rapidly increase in numbers in the susceptible animals and soon become detectable both within and outside the phagocytic cells. Whether bacterial multiplication occurs extracellularly, intracellularly, or both, is a question which has not been convincingly answered, and which cannot be discussed here.

During the past few months, my colleague, Dr. Hubert Bloch (17), has made a few observations which may contribute to the understanding of the relation of virulence to phagocytosis. By studying the exudate at intervals of time after injection of bacilli into the peritoneal cavity of mice, he could confirm that both avirulent and virulent forms were rapidly engulfed by phagocytic cells. He further gained evidence that engulfment of the virulent bacilli often resulted in the death and disruption of the phagocyte, a fact not observed with the non-virulent forms. These observations suggest the important conclusion that virulence is correlated with the ability of the bacillus to cause injury to the phagocytic cell. It seems legitimate to wonder whether this cytotoxic effect does not result in the liberation from the phagocytic cells of products that favor the further growth of the bacilli, a view for which suggestive evidence will be presented later.

Morphological and chemical differences between virulent and avirulent tubercle bacilli. Many efforts have been made to recognize between virulent and avirulent tubercle bacilli differences in their immunological or chemical characteristics, but with unconvincing results. On the other hand, bacteriologists have long been aware that certain morphological aspects of the bacterial growths appear to be correlated with virulence (18, 37a, 63). Without attempting to review earlier observations, I shall limit myself to a brief summary of the morphological characteristics recognized by Dr. Middlebrook in cultures of tubercle bacilli cultivated in the liquid and agar culture media developed in our laboratory (33, 59). In all virulent forms of human and bovine strains so far studied, the cells exhibit a marked tendency to adhere to one another in the direction of their long axis; this tendency results in the formation of strands of bacilli, which can be very long and at times extend over several microscopic fields, and which are several cells in thickness. In contrast to this serpentine pattern of growth, the avirulent variants exhibit either random growth or perhaps a rosette arrangement of the cells. These microscopic morphological differences are reflected in the macroscopic appearance of the cultures in liquid and on solid media, the virulent forms tending at first to give rise to thin films of growth spreading by the outward progression of the long strands of cells, whereas the non-virulent variants tend to multiply in the form of isolated islands consisting of heaped masses of bacilli (Plate I and Plate II). It is of interest that these fundamental morphological characteristics can be observed in animal tissues and therefore are not artifacts due to cultivation in unorthodox media (55). On the basis of present information, therefore, it appears worth considering as a working hy-

pothesis that the substance which tends to make the virulent bacilli adhere to each other and which causes their spreading mode of growth is in some obscure way correlated with virulence, perhaps by virtue of its ability to exert a toxic cytolytic effect on phagocytic cells.

Unfortunately, there is as yet no convincing information concerning the chemical nature of this substance and it can only be stated that the tendency of the virulent bacilli to grow in the form of "cords" is overcome by the addition to the culture medium of certain types of non-ionic wetting agents (33, 59). It is possible that the morphological differences between virulent and avirulent bacilli are not of a qualitative but only of a quantitative nature, and may be due to the production by the virulent forms of larger amounts of a certain hydrophobic substance, much less abundant in the avirulent form, which is wetted by the non-ionic surface active agents.

Toxic and allergic reactions in tuberculosis. Granted the ability of virulent tubercle bacilli to become established in susceptible hosts, knowledge of the nature of the disturbances by which they cause disease is far from clear and complete. The existence in tubercle bacilli of components capable of exerting a direct toxic effect has often been surmised, but has not been convincingly demonstrated (65, 68). The facts that undiluted tuberculin is somewhat toxic to cultures of normal tissues (2, 60, 66, 67, 69) and that killed bacilli, or extracts of them, can provoke the histological changes of tuberculosis (20, 71) are only questionable evidence of the existence of a toxin. There is no doubt on the other hand that tuberculous infections bring about a number of allergic reactions which are certainly of importance in the symptomatology and pathology of the disease. As is well known, the best studied among these is the allergy to bacillary protein which has been so extensively described under the name of delayed or tuberculin type of reaction. We need not review here the classical studies concerning the purified protein fractions which have been shown to possess a high degree of tuberculin activity (74). In addition to their great theoretical and practical interest, these purified proteins provide examples of convincing correlation between chemical nature and biological activity. It must be emphasized, however, that allergy in tuberculosis is not limited to the reactions to the purified proteins. Not only have other protein fractions been shown to exhibit tuberculin activity (56) but it is also certain that still other components of the bacterial cell (carbohydrates) can elicit allergic reactions of the immediate (anaphylactic) type (21, 56a). As far as is known, these reactions can be induced by virulent and avirulent bacilli alike. The capacity to induce the allergic state, therefore, cannot be synonymous with virulence, although it certainly plays an important part in pathogenicity.

It is one of the remarkable properties of tubercle bacilli that their mere presence greatly enhances the antigenicity of many unrelated substances (36). This is not a unique property of mycobacteria since, for example, antibodies against agar are often produced when other bacteria grown on agar gels are used for immunization. Nevertheless, the ability to enhance the antigenicity of diverse and unrelated substances is so intense in the case of tubercle bacilli that it must

have a bearing on the high level of allergy to their cellular constituents and products which accompanies tuberculosis; furthermore this property raises the possibility that immune reactions to some of the tissue constituents of the infected host may also take place during tuberculous infection. It is known that injection into rabbits, guinea pigs, and monkeys, of brain tissue mixed with heat-killed tubercle bacilli resuspended in paraffin oil brings about the production of antibrain antibodies and the development of allergic encephalitis (48, 61, 62). On the other hand, injection into rabbits of tubercle bacilli which have adsorbed certain synthetic surface active esters of oleic acid elicits the production of antibodies directed against these esters (58). As many types of surface active substances (phospholipids, cerebrosides, etc.) are widely distributed in tissues, and can become adsorbed on tubercle bacilli, one may wonder whether allergy to tissue constituents altered by the infectious process does not play a rôle in the pathology of tuberculosis. Whether these speculations are justified or not, it remains a fact that certain peculiarities in the cellular structure of tubercle bacilli render them specially effective in enhancing the antigenicity of many types of natural and synthetic materials, and it is very gratifying that progress is being made towards establishing the nature of the bacterial components responsible for this interesting property (20, 67).

Immunity to tuberculosis. That a limited, but unquestionable level of immunity to tuberculosis results from prior exposure to tubercle bacilli, is shown by epidemiological and clinical observations in man, and can be demonstrated in experimental animals. But no convincing theory has been offered to account for the mechanism of this acquired immunity.

As tuberculous infection brings about the state of tuberculin allergy, one of

PLATE I

1a. H37Ra. Ziehl-Neelsen stained smear of a 7-day-old culture in liquid medium containing 0.02 per cent 'tween 80' and 0.5 per cent serum albumin. Note the lack of orientation in the arrangement of the cells of this avirulent strain. × 1000.

1b. H37Rv. Ziehl-Neelsen stained smear of a 7-day-old culture in liquid medium containing 0.02 per cent tween 80 and 0.5 per cent serum albumin. This culture was recently isolated from an experimentally infected mouse. Note the tendency to the formation of cords. × 1000.

2a. H37Ra. 12-day-old culture on the surface of the agar medium containing 0.01 per cent tween 80 and 0.5 per cent serum albumin. The colonies are smooth surfaced, raised, and opaque. X 90.

2b. H37Rv. 12-day-old culture on the surface of the agar medium containing 0.01 per cent tween 80 and 0.5 per cent serum albumin. The colonies are flat and translucent, and have serpentine markings. × 90.

3a. H37Ra. 12-day-old culture on the surface of the agar medium containing 0.5 per cent serum albumin and no tween. Note the non-oriented structure of the colonies: the colonies are heaped-up and have little tendency to spread out over the surface of the medium. × 90.

3b. H37Rv. 12-day-old culture on the surface of the agar medium containing 0.5 per cent serum albumin and no tween. The colonies have a serpentine structure; cords are visible in the form of loops at the thin undulate margins; and they are flat because of their tendency to spread out over the surface of the medium. × 90.

The photographs were made by Mr. Joseph B. Haulenbeck.

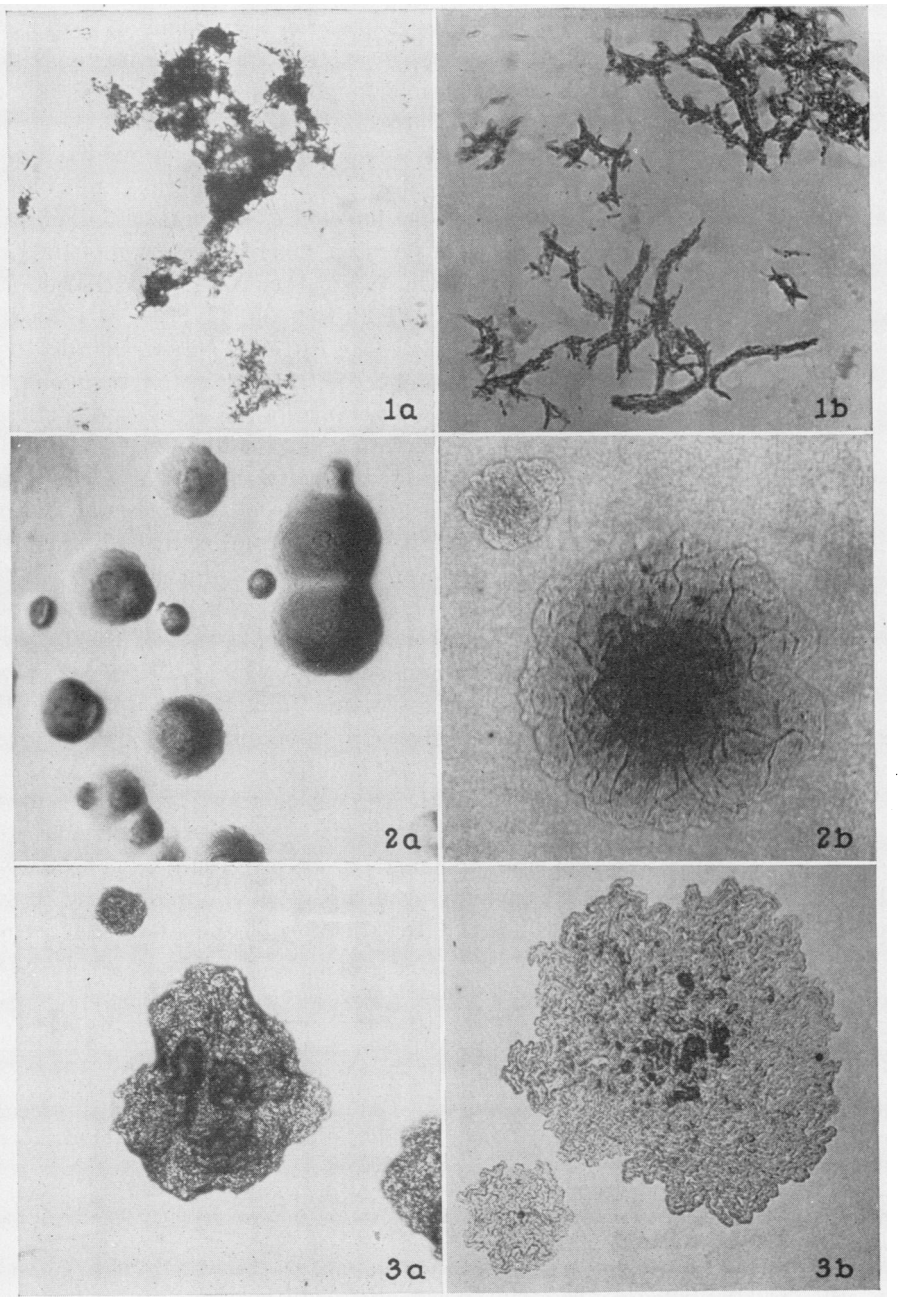

PLATE I

the possibilities which has been most widely discussed is that acquired immunity is a consequence, manifestation, of allergy. A brief statement of the known facts of allergy in pneumococcus infections may facilitate a more precise definition of this problem. As already mentioned, infection with, or injection of, pneumococci elicits multiple immunological reactions directed against the different antigenic constituents of the bacteria; several of these reactions have been recognized in terms of allergic manifestations. Thus, intradermal injection of the specific capsular polysaccharide into the infected or immunized animal calls forth an immediate, anaphylactic type of reaction, whereas injection of the bacterial nucleoprotein fraction or of the non-specific somatic polysaccharide (C) results in the delayed tuberculin type of reaction (12, 35, 47, 83). It is only in the case of the first of these three antigens (the capsular polysaccharide) that the allergic reaction depends on the presence in the serum and tissues of an antibody which is capable of protecting against infection; in other words, allergy to the capsular polysaccharide is correlated with a state of immunity. Whether in the case of pneumococcus, allergy means immunity, is therefore a question that can be answered only if one qualifies the nature of the bacterial antigen which is used to measure the allergic state. A similar situation may obtain in the case of tuberculosis. Although it has been shown that skin allergy (delayed type) to the tuberculoprotein does not necessarily reflect a state of immunity (65, 67, 68), it remains possible that there exist constituents of the tubercle bacilli against which is directed an immune reaction which can be recognized either by an allergy test or by immune resistance to infection. But, unfortunately, these bacterial constituents, and their corresponding immunological reactions, have not yet been identified.[4]

As the ability of the bacilli to exhibit the serpentine growth pattern seems to be correlated with virulence, it is enticing to believe that an antibody directed against the substance responsible for this mode of growth would have protective value. There is as yet no evidence in favor of this view; and in fact, the knowledge that immunity can be established by injection of the attenuated strain

[4] It should be pointed out at this time that the accumulation of inflammatory cells evoked at the site of the allergic reaction probably plays a rôle in modifying resistance, a fact which complicates the analysis of the relation of allergy to immunity.

PLATE II

1. Ziehl-Neelsen stained preparation of an 8-day-old culture of avirulent tubercle bacilli (H37Ra), grown in oleic acid albumin medium. The bacilli are not oriented and form clumps. × 1520.

2. Ziehl-Neelsen stained preparation of avirulent tubercle bacilli (H37Ra), grown in oleic acid albumin medium containing 0.5 per cent chick embryo extract. The bacilli are arranged in parallel and form cords. × 1520.

3. Ziehl-Neelsen stained preparation of virulent tubercle bacilli (H37Rv), grown in oleic acid albumin medium The bacilli form cords. × 1520.

4. Ziehl-Neelsen stained preparation of virulent tubercle bacilli, (H37Rv) grown in oleic acid albumin medium containing 0.5 per cent chick embryo extract. The cords are tighter than in fig. 3; the parallel arrangement is more pronounced. × 1520.

The photographs were made by Mr. Julian Carlyle.

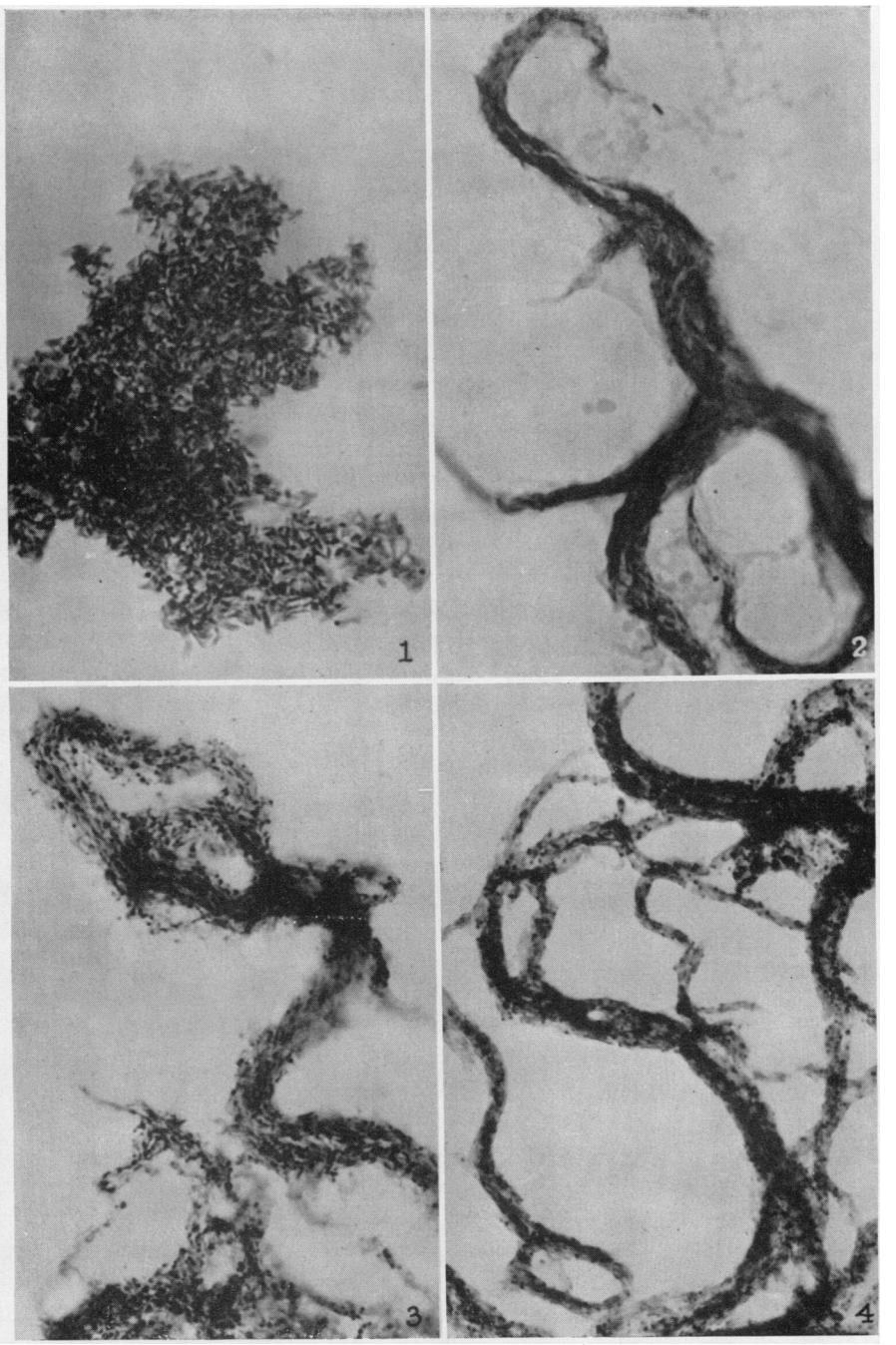

PLATE II

BCG would seem to militate against it. During the past few months, however, this objection has become less valid as a result of discoveries in the fields of pneumococcus and streptococcus immunity. There have been isolated a number of variant strains of these cocci which produce amounts of capsular polysaccharides (in the case of the pneumococci), or of the M proteins (in the case of streptococci) too small to allow bacterial proliferation *in vivo* and therefore too small to endow the bacteria with virulence, but sufficient to elicit the production of the homologous specific protective antibodies; in other words, there are known several strains of pneumococci and streptococci which have become stabilized at levels of production of the "virulence" antigens not adequate to permit progressive infection, but capable of calling forth the specific immune reaction (54, 70, 82).

It would be enlightening to reëxamine from this point of view the attenuated cultures of anthrax, fowl cholera, and swine erysipelas used by Pasteur in his celebrated experiments on immunization in order to determine whether they had retained some of their "virulence" antigens. For the immediate purpose of our discussion, in any case, it is worth considering the possibility that tubercle bacilli can also be stabilized at levels where they produce enough of the virulence antigen to immunize, but not enough to produce progressive disease. Of special interest in this respect is the fact that ability to exhibit the serpentine pattern of growth is not an all or none property in tubercle bacilli. On the contrary, strains differ quantitatively rather than qualitatively with reference to it, and, by adequate cultural techniques, it is possible to recognize that, in BCG cultures, the bacteria retain to some extent the ability to organize themselves according to this pattern, suggesting the existence in BCG of a small residuum of the virulence property (59).

Effect of in vivo environment on the antigenic structure of bacteria. The BCG vaccine consists of living organisms and its effectiveness as an immunizing agent depends upon its ability to achieve a limited degree of proliferation *in vivo*. This fact raises the possibility that the bacilli produce during growth in the animal tissues an antigen which is not produced *in vitro* in the ordinary culture media. There is a well established precedent for this hypothesis. In confirmation of Bail's early claims on the production of aggressins, it has been demonstrated during recent years that the edema fluid in animals infected with anthrax bacilli contains an antigen which is not produced by the bacteria in ordinary bacteriological media; this antigen is formed *in vitro* only under very special cultural conditions (39, 45, 85). There is as yet no evidence for the occurrence of a similar phenomenon in the case of tubercle bacilli, and the fact that it is possible to establish with vaccines made up of heat killed virulent bacilli a level of immunity equal to that induced by BCG suggests that the immunizing antigen is readily produced by the bacilli *in vitro*. Nevertheless, recent observations suggest that the morphology of attenuated tubercle bacilli, and perhaps therefore their antigenic constitution, can be modified if not qualitatively, at least quantitatively, by growth in the presence of certain components of animal tissues. Thus, Dr. H. Bloch has found that addition of aqueous extracts of chick embryo

to oleic acid-albumin medium causes non-virulent tubercle bacilli to exhibit the serpentine pattern of growth characteristic of the virulent forms, and that the same extracts also affect the virulent bacilli in the same direction, inciting them to grow in the form of bacillary cords much tighter than those obtained in ordinary media (Plate II). No technique is available to translate these findings into immunological terms. Nevertheless, preliminary observations suggest that the chick embryo extract has an enhancing effect on some aspect of virulence of the organisms growing in its presence; this effect however is entirely reversible, as the bacilli return to their original level of virulence as soon as transferred to media devoid of the extract (16).

In the past, the effect of the *in vivo* environment has been considered chiefly from the point of view of classical immunity reactions. It is important to remember, on the other hand, that many metabolic phenomena which must play a part in pathogenicity, are markedly altered by environmental changes. It is sufficient to recall in this respect that slight modifications in the composition of the medium, which have little if any effect on the rate of bacterial growth, increase or decrease out of all proportions the production of toxins by diphtheria bacilli, clostridia and other pathogenic agents (14). Similarly, the production of certain enzymes is an adaptive process, and depends upon the presence in the culture medium of the homologous substrate (28, 38). Although only two enzymes of interest in pathology (hyaluronidase and the lecithinase α toxin of clostridia) have so far been shown to exhibit adaptive behavior, the phenomenon is certainly one of wide occurrence and cannot fail to be of significance in pathological processes. The effect of the *in vivo* environment on the cellular structure and metabolic equipment of bacteria, and therefore on their pathogenic behavior, is one of the virgin fields of medical bacteriology.

Factors which affect the proliferation of tubercle bacilli in vivo. In the immunized or naturally resistant host, virulent tubercle bacilli may survive for prolonged periods of time, although they fail to increase in numbers. It is certain that under body conditions the bacilli respire and metabolize, but the nature of the bacteriostatic and bactericidal influences which control their multiplication is entirely unknown. Analysis of this problem will require eventually some knowledge of the metabolic reactions used by the bacilli for multiplication *in vivo*. As is well known, virulent tubercle bacilli can grow *in vitro* in synthetic media of extremely simple composition, but this tells us little of the substrates which they *do* utilize in the animal tissues. The observations made in our laboratory have led us to emphasize the rôle of lipids in the nutrition of these bacteria (29, 32), but it has not yet been proven that this rôle is as important in the body as we find it interesting in our test tubes. Nevertheless, there is no question that lipids play a peculiar rôle in the metabolism of tubercle bacilli. Thus it is a remarkable fact that, under the proper conditions, certain long chain fatty acids and alcohols, and even hydrocarbons, are much more effective than glucose or glycerol in stimulating their respiration (44, 51, 72). Furthermore, the yields of avian and human mycobacteria grown in serum albumin media containing palmitic, stearic or oleic acids (either in the form of the sodium soaps or of the water

dispersible esters), linoleic, linolenic or arachidonic acids, lecithin, kephalin, sphingomyelin or lignoceric acid, increase in direct proportion with the concentration of the fatty acid in the medium (29, 32).

Evidence is now accumulating that certain tissue components are capable of exerting a stimulating effect on the growth of tubercle bacilli. For example, the phospholipid sphingomyelin permits the growth of small inocula even in the absence of serum albumin and at the same time increases markedly the yield of growth within a given period of incubation (32). This dual effect can be analyzed in terms of two independent properties. On the one hand, sphingomyelin, like albumin, can detoxify long chain fatty acids and thereby exerts a protective effect on the bacilli. On the other hand, it acts as a source of nutrient probably by virtue of the lignoceric acid which is present in amide form in its molecule. Other tissue factors present in aqueous extracts of chick embryo, and as yet unidentified, have also been found to enhance and modify the growth of tubercle bacilli *in vitro* (16, 37, 76a). It appears likely, therefore, that as more is learned of the requirements of these organisms, it will become possible to reduce considerably their generation time *in vitro*, a result which would be of theoretical and practical importance especially in view of the fact that the generation time of tubercle bacilli *in vivo* appears under many conditions to be much shorter than is usually assumed.

Although serum is a poor medium for the growth of tubercle bacilli, their growth is abundant when the cellular elements of the blood have been lysed (66a, 88). These facts suggest the possibility that the growth promoting effect of sphingomyelin and of substances similar to those present in embryo extract may have a bearing on the problem of bacterial proliferation *in vivo*, particularly in caseous material and in the necrotic tissue found in silicosis. The relation of the cytotoxic properties of virulent tubercle bacilli to the local release of cellular components available for the nutrition of the bacteria is also a subject worth meditation and experimentation.

The inhibition of growth by immune processes directed against the metabolic enzymes of bacteria has often been postulated. "Antiblastic immunity" was first mentioned in the case of anthrax by Ascoli (3) and later in the case of pneumococcus infection by Dochez and Avery, possibly the basis of mistaken interpretation of accurate experimental findings (15, 25). The concept of antiblastic immunity is compatible with the knowledge that antibodies can be produced against certain enzyme proteins and can inhibit their enzymatic activity (75). The recent finding that, in paramecia, specific antibody not only abolishes motility, but also brings about a hereditary alteration of the antigenic structure of the cilia, (76b), demonstrates that immune reactions can affect the course of the enzymatic reactions which determine the composition of microbial cells. At the present time, however, factual evidence that there exist immune humoral factors which can inhibit the proliferation of parasites has been obtained only in the case of infection by certain protozoa and helminths (81). Whether the "anablastin" demonstrated by Taliaferro and his colleagues is really an antibody and whether it has its counterpart in tuberculous disease are questions which cannot be answered at the present time.

Finally there remains to be mentioned the possible existence in tissues of substances other than immune bodies capable of exerting a bacteriostatic or bactericidal effect on growth. Thus, long chain fatty acids are extremely toxic for tubercle bacilli, and although their toxic effect is overcome under ordinary circumstances by serum albumin and sphingomyelin (13, 22, 23, 24) there may be circumstances, in autolyzing tissues for example, where their bactericidal effect can come into play. Sphingosine may also be toxic for tubercle bacilli (32). In fact, it is becoming apparent that the resistance of tubercle bacilli to antibacterial agents has been greatly exaggerated. Not only are mycobacteria normally susceptible to many more agents than was formerly suspected, but it is also possible to increase their susceptibility by slight changes in the environment. Thus, all surface active substances which facilitate dispersed growth of tubercle bacilli by wetting their hydrophobic surface increase the susceptibility of these organisms to a variety of antibacterial agents (triphenylmethane dyes, *p*-aminosalicylic acid, streptomycin, subtilin, penicillins) (30, 49, 50, 79). For example, strains of mycobacteria which can grow in oleic acid albumin medium (in which their surface is strongly hydrophobic) in the presence of 100 micrograms of penicillin or of subtilin per milliliter, are inhibited by 5 micrograms of these inhibitors in media containing the proper wetting agent. Whether this is due to the more dispersed state of the cultures in media containing the wetting agents or to the fact that the latter substances facilitate access of the inhibitor by modifying the bacterial surface is as yet unsettled. In any case, the important conclusion can be drawn from these facts that the resistance of tubercle bacilli to inhibitors which are effective against other microbial species need not be the consequence of peculiarities in the metabolic equipment of the former organisms. It may be due merely to the hydrophobic character of their surface which retards or prevents the contact between the inhibitor and the susceptible cellular substrate. Although these results were obtained in artificial media, and with synthetic wetting agents, it is worth keeping in mind that animal tissues also contain many types of surface active agents which, under certain circumstances, may alter the susceptibility of tubercle bacilli to various agents.

Whatever the real nature of the mechanisms which hold in check the increase in numbers of tubercle bacilli in the infected individual, they often result in a delicate equilibrium between parasite and infected host. But this equilibrium is extremely unstable, and it is well known that many changes in the host or his environment can bring about reactivation of a dormant tuberculous infection. To illustrate this statement, it is sufficient to recall how suddenly tuberculosis mortality rates increased in different parts of the world during the first and second World Wars. Within a few months, tuberculosis had taken the lives of many individuals who, prior to the upheavals associated with the war, were living in a state of unsteady equilibrium with their disease (1a, 87). These observations provide fertile hunting ground for the epidemiologist. From our point of view, they emphasize once more the necessity of identifying those structures and reactions at the level of which the animal and bacterial economy influence each other.

Conclusion. We have travelled a long way from the analysis of the rôle played by capsular polysaccharides in the pathogenesis of pneumococcus infections, but

a single thread has led us through our wanderings. We have attempted to analyze the ability of microbial parasites to invade and cause disease, and the immunological and pathological responses of the infected hosts, in terms of the rôles played by the different constituent parts of the parasitic cell.

The results obtained in the study of pneumococcus immunity remain as a symbol of perfection and as a goal in our efforts. Even today, after twenty years of experimentation in all fields of medical microbiology, no experiment compares in elegance and convincingness with the demonstration that one can bring about specific immunity against pneumococcus infection by injecting the purified capsular polysaccharides into experimental animals (41, 42, 43, 73) or man (35, 53). Indeed, it has been found possible to establish a certain level of immunity against virulent pneumococci by means of synthetic antigens which mimic the immunological specificity of the capsular polysaccharides, although they do not contain any part of them (40). The analogy with the evolution of our knowledge of hormones and vitamins is worth bringing out at this time. Following the recognition by physiological methods of hormonal influences and vitamin deficiencies, the chemist, guided by precise biological tests, identified and synthesized the active chemical agents. A similar process is slowly emerging in the study of infectious diseases, and Dr. Avery's work has historical significance because of his pioneer and unexcelled contributions in this field. It was he, who first established experimentally the importance of identifying the structures and functions of microbial cells which are of significance in the infectious process. With him, microbial parasitism evolved from an ecological concept into a body of facts and doctrines which define in physicochemical terms the mechanism of host parasite relationships.

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