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Growth Habits Of *Mycobacterium leprae* Their Implications

B. R. Chatterjee, M.B., B.S.[1]

Although *Mycobacterium leprae* was among the first microbes to be described as associated with disease, we were largely ignorant until recently about its basic biologic characteristics. During the last two years, I have been engaged in renewed efforts to cultivate *M. leprae in vitro,* guided largely by new concepts on bacterial persistence developed through studies by myself and others. I have also received valuable suggestions from the experience of my colleagues on the growth of chelate-requiring mycobacteria. Dr. Hanks has been a constant counselor on all matters and a source of ready reference. This presentation will summarize briefly new findings and concepts regarding two areas of research, viz., (1) the proclivity of *M. leprae* to grow both *in vitro* and *in vivo* in weak-walled and transitional L forms, and (2) the implications of these findings with respect to the insidious onset and latency of leprosy.

First, I would like to dwell briefly on the background information that led to the type of approach used in this study. Earlier cytologic studies by myself and R. P. Williams (3) demonstrated that degenerating bacteria in old cultures are not really dead or defunct. On the contrary, such cells centralize their resources and genome in small loci called chromatin bodies or nucleoid bodies. These bodies could be isolated from autolyzed cultures or liberated from viable cells by lysozyme digestion of cell walls in media of high osmotic pressure. In ordinary bacteriologic media they failed to grow. But in media containing rich nutrients, PPLO serum fraction, or horse serum, and high salt concentration, they grew as L forms, and occasionally reverted to bacilli indistinguishable from the parent *B. megatherium.*

Although many related observations have been made with mycobacteria, the significance of Much's granules and the filterability of the subbacillary forms would have to be reinvestigated by modern methods in order to clarify the controversies they have caused. Brieger and associates (2), however, have clearly demonstrated that early phases of bovine tubercle bacillus infection in rabbits occur by means of non-acid-fast, noncultivable particles. Mattman *et al.* (8) have reported that L forms can be induced in mycobacteria and occur during mycobacterial infection in man. It is now known that the small mycobacterial granules and elements of the L forms can pass through filters that hold back bacteria. Furthermore, in wet preparations of normal cultures of mycobacteria, after vigorous shaking by hand, small nonacid-fast granules can be found routinely. Although this factual background at the outset of my experiments with *M. leprae* has more relevance to subbacillary forms of bacteria, my aim was to cultivate the bacillus. Isolation of the nucleoid bodies from *M. leprae,* and growth as L forms, were not considered feasible at that time.

Before describing the forms in which *M. leprae* might grow *in vitro,* I would emphasize that all inocula consisted of rods. These were separated from the soft-walled growth forms in tissues by washing in water and by enzymatic digestion. Successive treatment with pancreatin, lipase and Pronase (from *Streptomyces griseus*)[2]

[1]Johns Hopkins-Leonard Wood Memorial Leprosy Research Laboratory, Baltimore, Md. Present address: All India Institute of Hygiene and Public Health, 110 Chittaranjan Ave., Calcutta, India.

[2]Calbiochem, 3625 Medford Street, Los Angeles, California 90063.

eliminated tissue components as far as possible, and digested the elements of the L forms. After count of the bacilli by the method of Hanks *et al.* ([7]), approximately 5 x 10^6 bacilli per ml. of medium were inoculated. The first nutritional base that proved of interest included Bacto penassay broth, with 20 per cent Difco PPLO serum fraction. Because of the great variety of growth forms that occurred, the true extent of replication of genetic units and cytoplasm could not be judged. Some of these forms, however, succeeded in producing complete rods. The net increase was on the order of 10-fold.

In further work it has been learned that improvements in bacillary yields are obtained at pH 5.5 and by the use of 10 per cent CO_2, conditions suggested by the work of my associates on the chelate-requiring mycobacteria ([4,9,11]). Higher yields of bacilli are obtained at 32° than at 34° or 37°C. Many comparisons of nutritional bases have not confirmed the usefulness of the Watson-Reid synthetic base. High concentrations of sodium chloride used in the earlier experiments to give osmotic protection to the L form elements, were found to be unnecessary and inhibitory. At present the best results are obtained in trypticase-soy broth (BBL), supplemented with 20 per cent horse serum or 2 per cent human serum, in basal salts solution. By means of these procedures, the average yield of complete bacilli has been increased to 30-40 times the starting number.

I will first describe the macroscopic evidence of continued synthetic activity within these cultures. Otherwise, the nonquantitative description of cytologic forms has no meaning. After about 2 weeks a perceptible granular sediment begins to accumulate on the bottom of the tubes. Irrespective of the growth forms, the volume of this fluffy sediment continues to increase until in 4- to 6-month-old cultures studied to date, it may occupy approximately a third of the total depth of the medium. This fluff does not occur in uninoculated or control media. Varied cytologic elements contribute to the accumulation, but the predominant elements are clusters of small granular forms typical of transitional L forms. These changes, and multiplication of rods and L forms, do not occur in conventional media used to cultivate mycobacteria. Further, in the same medium, but at a pH of 7.0 and at 37°C, these changes occur only at a very slow rate.

I will not describe the sequence and variety of cytologic forms that occur in these cultures. Within the first week of incubation, the chromatin bodies inside the bacilli enlarge with stretching and disappearance of the cell wall. At the end of 2 weeks, spherical bodies, occurring singly or in clumps, are released into the medium. At about this time a perceptible granular sediment accumulates at the bottom of the tubes. After 3 to 4 weeks of incubation, the spherical bodies enlarge considerably and tend to agglomerate into large clusters. Many of these bodies develop smaller spherical elements or bacilli, or a combination of both elements inside of these. This process proceeds slowly and may go on for 6 to 10 weeks. Because of this mixture of spherical forms and complete bacilli, the major growth at this time can be defined as transitional L forms. There is concomitant increase in the granular sediment in the tubes. If sampled with minimal agitation, microcolonies of L forms are found. Simultaneously, a different type of cellular organization takes place. Small and medium-sized spherical bodies occur on long filamentous processes like "peas in the pod." These filamentous processes break down subsequently during 8 to 12 weeks of incubation, releasing small and discrete chromatin-like particles. Although the peak of bacillary multiplication occurs in 4 to 6 weeks, further production of rods and L forms goes on for months in the culture supernatant after the organized elements are separated out of the tubes by centrifugation. But this process is very slow. The spun-down growth, however, does not multiply further on addition of fresh medium. Besides these productive elements, large numbers of highly plastic membranous bodies that look like amebae are found. These forms fail to produce bacilli inside of them. There occur also unorthodox forms of multiplication. After 10 to 12 weeks, the fluffy, granular sediment

described before begins to accumulate. All these forms are highly susceptible to drying, fixation, and staining, and can be observed only by phase contrast or dark field examination. The next few slides show some of these forms that have retained their shape and structures after Ziehl-Neelsen staining. Growth of similar nature was observed by Becker and Brieger ([1]) in an organ culture system, and by Dr. Claude V. Reich in the Philippines ([10]) in a paper that you have already heard.

Certain of the growth forms that develop *in vitro* are valid counterparts of the growth of *M. leprae* in tissues of lepromatous patients. The main difference is that the large spherical bodies in the tissues are larger and more densely packed with bacilli. There is little doubt that these are present as globi, after the membrane is destroyed by staining. I might add that the membrane-bound organization of the globus was suspected by Hansen in his early studies, and later by Denney in 1934 ([5]). The significance of this observation could not be appreciated in those times, and the observations were not followed up by any other investigator.

The extent of multiplication of genetic units is greater than can be gauged by increase in bacillary numbers. A larger proportion of the new growth occurs as weak-walled or L forms that are susceptible to staining procedures and can be seen only in wet mounts. Therefore, the problem of quantitating the extent of growth as rods and L forms separately remains unsolved. Optical density measurements are not satisfactory because of the complexity of the inoculating material.

DISCUSSION

It is not possible to discuss all the implications of these findings in a short time. I will confine the discussion therefore to a few important areas.

Hanks ([6]) demonstrated that complete cells of noncultivated mycobacteria are remarkably impenetrable. *M. leprae* gets around this defect through growth as wall-deficient L forms or other weak-walled forms. Also, simultaneous growth of large numbers of rods inside the L bodies explains the rapid proliferation of bacilli in fast-progressing lepromatous leprosy and during reactional states. Such phenomenal increase in the number of bacilli cannot be accounted for by binary fission only, with a generation time of 2-3 weeks.

The toxicity of bacterial pathogens and the pathologic change it produces is due mostly to the components of the bacterial cell wall. Having no cell wall, the L forms of bacteria are incapable of producing gross pathologic changes and clinical disease. It is only when these wall-deficient forms revert to normal bacterial forms that they acquire toxicity and pathogenic properties and cause clinical disease. This difference in the properties of L forms and their bacterial parent has been well established in clinical infection by studies of Wittler *et al.* ([12]). These studies have also shown that administration of penicillin and artificial immunization prevent reappearance of the complete bacterial forms, although the transitional L forms persist in the host. Such periods of induced latency coincide with subsidence of clinical symptoms, and withdrawal of the antibiotic would cause reappearance of clinical disease. Insidious onset, periods of latency or quiescence interrupting the course of the disease, and relapse or recrudescence after cessation of chemotherapy, are well known in leprosy.

In view of the experiences stated above and the finding of the L forms of *M. leprae* in tissues of the host, it is safe to assume that these phenomena are the result of maintenance of the disease initially in the transitional L form state. As long as the host affords varying degrees of resistance or immunity to the disease, the transitional L forms are unable to revert to the bacillary forms, or do so only at a low rate. Thereby, the disease maintains a subclinical, latent, or benign course in the host, difficult to detect by known clinical methods. Any change in the immune status of the host would trigger off the return of complete bacilli from the L forms and cause an explosive onset of the disease. The same could be said of persistence of the disease and relapse after years of chemotherapy. It

is possible that the sulfones, while inhibiting the turnover of new bacilli by suppressing the reversion from L forms, cannot eliminate the transitional L forms. Persistence of these forms in the host can bring about relapse by reversion to bacillary forms.

Simultaneous lysis or rupture of large numbers of L forms, releasing large amounts of soluble or particulate antigens, could upset the immune balance of the host and precipitate reactional states.

Finally, I would like to comment on the developing trend of reliance on the elimination of solid-staining bacilli as an index of the success of chemotherapy. First, there are patients who have not received any chemotherapy who show no solid-staining bacilli. Second, by emphasis on solid-staining bacilli, we ignore the vast spectrum of bacilli that are not solid-staining but not degenerate either. It is beyond question that the solid-stained bacilli are the most rugged ones, capable of withstanding the rigors of exposure during their journey from the diseased to the healthy host. I trust that this study has shown that the granulated and other forms of apparently degenerate bacilli in the tissues should not be ignored or treated as harmless. In conclusion, I feel that these observations open up other possibilities for exploring and explaining certain of the fundamental features of the disease. To the microbiologist, the problem of getting the L forms to grow as complete bacilli *in vitro* remains a challenge. Finally, I should say that although this is an entering wedge, it has been evident that the conditions or factors required for successful cultivation have not been defined.

SUMMARY

Mycobacteria obtained from lepromatous nodules when incubated in a highly nutrient medium containing serum or a serum constitutent (Difco PPLO Serum Fraction), go through a phase of cell-wall-deficient forms that resemble the L form cycle in bacteria. The basis of this conclusion is the morphologic characteristics of the forms observed in the culture medium when wet mounts are examined by the phase contrast microscope. There is a proliferation of the acid-fast bacillary forms to the extent of 30-40 times after 6-10 weeks of incubation. This does not indicate the extent of total multiplication of bacterial substance, because the elements of the L forms are destroyed by the staining process. Physical conditions found most helpful for this phenomenon are a pH of 5.5, incubation at 32°C, and a concentration of CO_2 under 10 per cent. This multiplication either does not occur or occurs very slowly in conventional media or cultural conditions. It has not been possible to obtain growth on transfer or by replenishing the cultures with fresh medium. Valid counterparts of these morphologic forms are found in lepromatous tissues when a suitably prepared wet mount is examined by the phase contrast microscope. The significance of these observations is discussed.

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DISCUSSION

Dr. Rees. Thank you, Dr. Chatterjee. Now I will call on Dr. Ruth Wittler, Chief of the PPLO Research Section, Walter Reed Army Medical Center, to open this discussion.

Dr. Wittler. The demonstration by Dr. Chatterjee of the L form of *Mycobacterium leprae*, both in culture and in lepromatous tissue, has aroused widespread interest.

All of us who have been engaged in L-form research have noted the increasing frequency with which L forms are being found in infections. We ask ourselves if *in vivo* L forms are something new and perhaps related to modern prophylactic and therapeutic methods, or if they have always occurred in disease but simply eluded our recognition. In the light of Dr. Chatterjee's findings, the latter alternative now appears more probable. If L forms arise in a disease as ancient as leprosy, we have some justification in assuming that L-form variation is an inherent and basic property or potential of the organism itself. It seems probable that *M. leprae* has always been able to employ this prerogative to survive in the host environment down through all the centuries of its existence.

That you may appreciate more fully the implications and significance of Dr. Chatterjee's work, I would like to review briefly what we know of the discovery, the nature, and the behavior of L forms of bacteria. The first L form known was isolated in 1935 by Emmy Klieneberger from a culture of *Streptobacillus moniliformis*, the rat-bite-fever bacillus. At first she thought she had found a new pleuropneumonia-like organism (or PPLO) that lived symbiotically with the streptobacillus. She named the new organism "L_1," "L" for Lister Institute, where she was working, and "1" for the first such organism isolated there. Like the PPLO, the L_1 reproduced via minute granule-like cells that were filterable but grew on serum-enriched agar. They formed characteristic colonies with central cores composed primarily of granules which grew deep into the agar. The peripheral portion was spread out on the surface of the agar and was composed of large, fragile, pleomorphic bodies, intermixed with granules, bubbles and vacuoles. Figure 1 illustrates the typical appearance of an L-form colony.

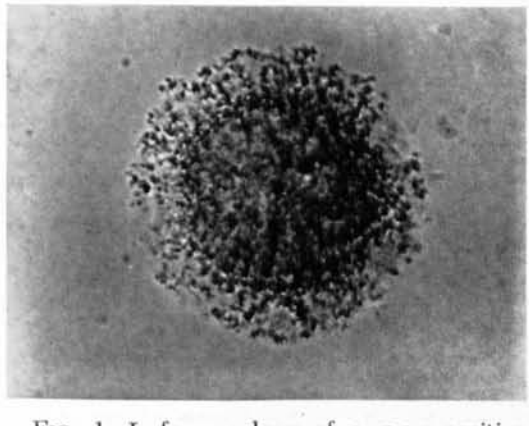


Fig. 1. L form colony of a gram-positive coccus. Magnification ×576.

Louis Dienes soon showed convincingly that the L_1 was not a symbiotic PPLO but a growth variant of the *streptobacillus* parent organism itself. He then proceeded to show that in the presence of high-salt concentrations, immune serum and complement, or penicillin, L forms could be derived in the laboratory from many other bacterial genera. The L forms of totally different genera all appeared similar in morphologic structure; all lacked cell walls, and all were gram-negative. Although their phenotypic characteristics frequently differed from those of their bacterial parents, the fact that L forms could be made to revert to their normal bacterial form gave proof of their unaltered genetic identity. Two types of L forms were recognized: unstable ones, which reverted to bacterial form as soon as inhibitors, such as salt or penicillin, were removed from the culture medium, and stable ones that no longer reverted even in the absence of inhibitors.

In the beginning, L forms were thought to be strictly a laboratory phenomenon. Stable L forms were not isolated from diseased hosts, and when a stable L was inoculated experimentally, it always proved nonpathogenic. It was several years before we suspected that the unstable L forms were the ones that arose spontaneously in the infected host and that these did play a role in the disease process. In fact, in the infected host, we found that there was a whole range of nonfixed, nonbacterial phases, including granules, large bodies and amorphous phases, as well as the typical unstable L phase, in which an organism could survive, multiply, and preserve its genetic identity with its parent bacterium. For convenience we have termed all these variant morphologic forms the "transitional forms" of a bacterium.

In 1957 our laboratory began to explore the when, where, and why of L form and transitional form production in human disease. One case in particular revealed a pattern that has been helpful for further investigations in this field. The patient in this case was a four year old girl with an interventricular septal defect and subacute bacterial endocarditis. Blood and bone marrow cultures taken upon admission to the hospital and before the start of antibiotic therapy yielded a microaerophilic *Corynebacterium* sp. Under penicillin therapy, clinical symptoms subsided and bacilli disappeared from the blood, but unstable L forms and transitional forms of the organism appeared and persisted in the host. Each time penicillin treatment was interrupted, the patient suffered a recurrence of fever and illness, and the bacillary form of the organism reappeared in her blood. Thus it seemed that the antibiotic-resistant transitional form was associated with latent stages of the infection, and that the antibiotic-sensitive *corynebacterium* was associated with active stages of the infection.

In the patient's blood we found masses of small granular inclusions in the cytoplasm of the monocytes (Fig. 2).[1] We be-

[1]Figures 2 to 9 were published in the *Journal of General Microbiology* (23 (1960) 315-333), and are here reproduced with that journal's permission.

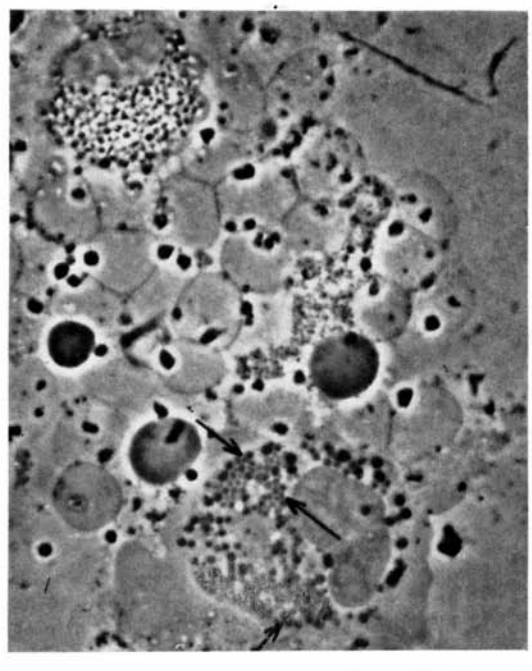


FIG. 2. Small round inclusion bodies (indicated by arrows) in the cytoplasm of WBC of the patient's blood. In the upper left corner of the photograph is an eosinophil containing the denser and highly refractile normal cytoplasmic granulation for comparison. Dienes' stain and phase contrast. ×1520.

lieve these are the transitional forms of the organism as they occur *in vivo*. When blood specimens containing these inclusions were cultured, colonies of granules, which penetrated deep into the agar, developed in 5-7 days (Fig. 3).

On occasion, blood cultures yielded not only the granular cores but large L colonies with their typical spreading periphery (Fig. 4). The hole in the center of the colony in the figure was filled with the deep granular core, which remained in the agar when the slide was impressed on the surface.

Figure 5 shows clusters of the large round body variant that developed in broth cultures of the patient's blood. Figure 6 shows the beginning breakdown of the round bodies, with release of the small granular forms. This is similar to a stage in the *M. leprae* L cycle. Figure 7 shows the final stage of breakdown of large bodies with granules embedded in an amorphous matrix. This again is similar to a stage of the leprosy L cycle. Figure 8 shows beginning reorganization in the amorphous mass. The granules begin to line up as beads on delicate filaments. Again, this is similar to the reorganization of *M. leprae*. Figure 9 shows the final stage in reversion, when the newly formed bacilli emerge from the reorganized masses.

In this case surgical repair of the septal defect was clearly desirable, and yet persistence, in the blood, of an organism

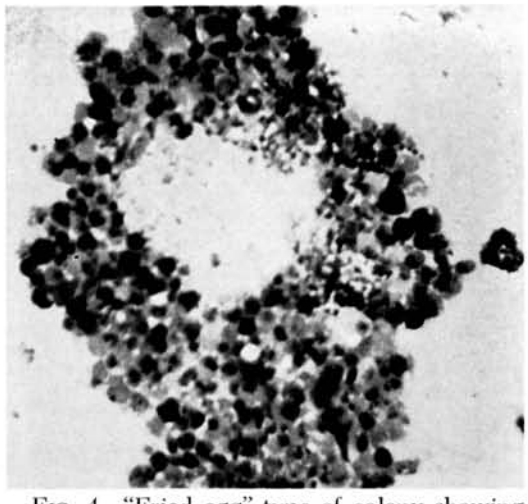


FIG. 4. "Fried egg" type of colony showing large bodies, vacuoles and granules which made up the peripheral portion of the colony. The granular central portion of the colony remained on the agar when the impression preparation was made, thus leaving an empty hole in the center of this specimen. Giemsa stain. ×1620.

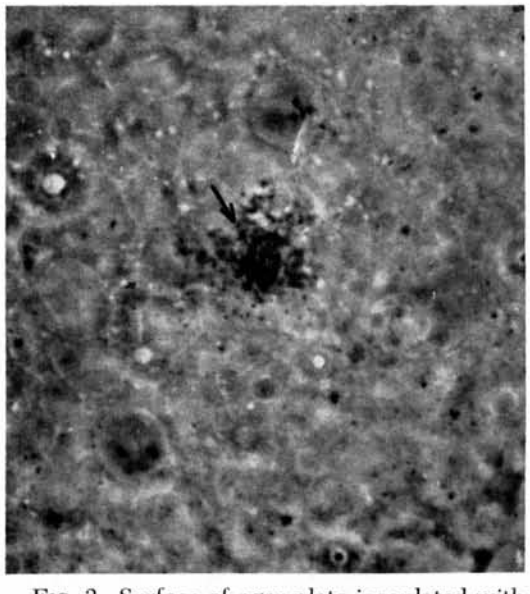


FIG. 3. Surface of agar plate inoculated with patient's blood showing the debris of distintegrated WBC after 5 days of incubation with the focus adjusted 8-10μ below the surface of the agar. The arrow indicates the deep colony of granules of most dense growth. Dienes' stain and phase contrast. ×1520.

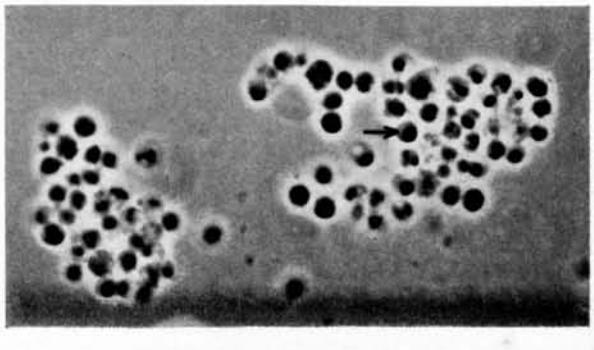


FIG. 5. Large clusters of round bodies. Some show dark caps on a clear bubble; others show differentiation of internal contents; still others are breaking down and merging into delicate masses of amorphous material. Four-day-old culture in VIB + Th(BBL) + AscFl + penicillinase. Hanging drop, unstained, phase contrast. ×1544.

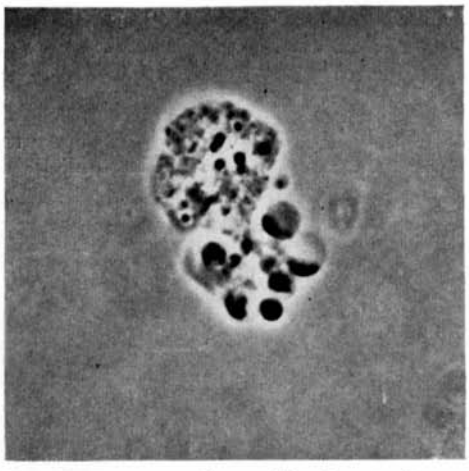


FIG. 6. A group of round bodies in the process of breaking down and merging, and the development of an amorphous mass filled with granules of various sizes. Sixty hour culture in PPLOFl + AscFl + penicillinase. Hanging drop, unstained, phase contrast. ×1781.

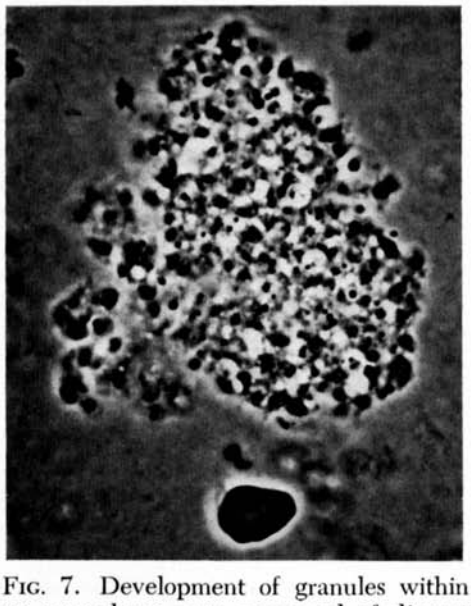


FIG. 7. Development of granules within a large amorphous mass composed of disrupted bodies, structureless protoplasmic material, and bubbles. Four-day-old culture in Th(BBL) + rabbit S. Hanging drop, unstained, phase contrast. ×1544.

known to be capable of reversion to a more pathogenic form, posed a grave risk. We theorized that if we could stimulate the host's antibacterial defenses and depend on these, as well as on the antibacterial action of penicillin, surgical risk might be minimized.

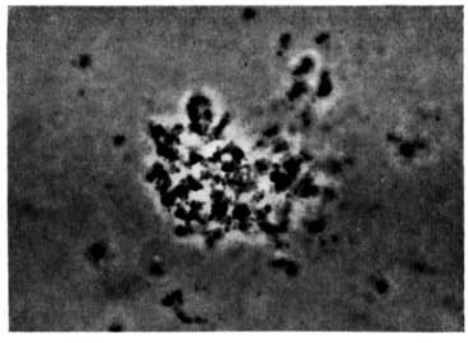


FIG. 8. A stage shortly before reversion to bacilli. The granules become arranged in orderly fashion and appear as beads on an extremely fine thread. Culture same as Figure 6. Agar block, Dienes' stain, phase contrast. ×2375.

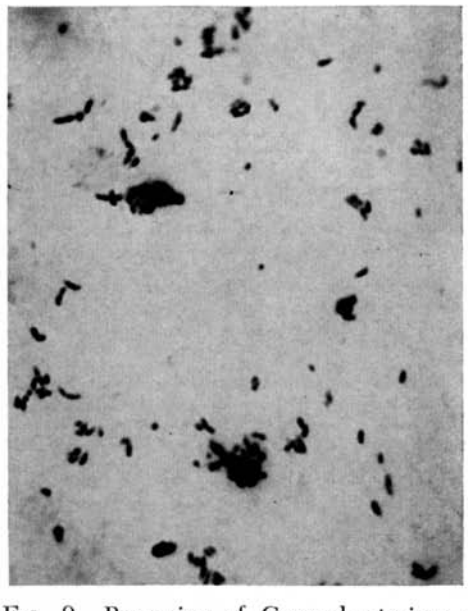


FIG. 9. Reversion of Corynebacterium sp. (Strain 11-4d) from transitional form. Eleven-day growth in Th(BBL) + rabbit S inoculated with the patient's blood. Giemsa stain. ×1620.

A heat-killed vaccine was, therefore, prepared from one of the child's own corynebacteria which had been isolated in its transitional form and reverted to the bacillus. Penicillin therapy was discontinued for almost three weeks before starting the vaccine. When the patient became febrile and a relapse seemed imminent, penicillin was resumed and continued until she recovered. Then the vaccine was started at

once, and shortly thereafter penicillin was discontinued without ill effects. The vaccine was given subcutaneously in small increasing doses for nearly one year.

The patient's serum had always been negative for complement-fixing antibodies to her corynebacterium, and in spite of vaccination this reaction remained negative. About 10 weeks after the start of vaccine therapy, however, the patient developed positive serum reactions for rheumatoid factor and nucleoprotein antibody. After several more months of vaccine therapy her serum again became negative for these hypersensitivity or auto-immune reactions.

Bacteriologic studies revealed that the granular stage of the organism remained in the child's blood even after six months of vaccine therapy, but reversion to the bacillus could no longer be obtained by any of our cultivation methods. Open-heart surgery to repair the septal defect was finally undertaken five years after the onset of endocarditis. Vaccine was administered until the time of the operation, and large doses of penicillin given before, during, and after the operation. No vegetation were found in the heart, surgery was successful, and the patient recovered.

In other bacterial infections the presence of L forms is now being demonstrated. Godzeski *et al* (Nature **205** (1965) 1340) and our own laboratory have isolated L forms of staphylococci from various human chronic staphylococcal infections. Mattman *et al.* (*American Rev. Resp. Dis.* **82** (1960) 202) have isolated *M. tuberculosis* L forms from the cerebrospinal fluid of patients with tuberculous meningitis. In the laboratory, inoculation of animals with various bacteria can result in conversion *in vivo* to the L form, and from these experiments we begin to see the conditions under which L forms evolve. Many years ago I demonstrated conversion of virulent *Bordetella pertussis* to the L form in the lungs of immunized mice; in nonimmune mice the organism remained in its bacillary form and rapidly killed the host (*J. Gen. Microbiol.* **6** (1952) 311). Guze and Kalmanson (*Science* **143** (1964) 1340), studying enterococcal pyelonephritis, found that in the kidneys of penicillin-treated rats *Streptococcus faecalis* underwent a change to a large round body or protoplast form. Mortimer (*Proc. Soc. Exper. Biol. & Med.* **119** (1965) 159) showed that strains of *Streptococcus pyogenes* of moderate to low virulence, when inoculated into normal mice, converted to L forms, and stable L-form colonies were obtained upon culture. Virulent streptococci showed little conversion to the L form in normal mice. Thus there is experimental evidence that natural or acquired resistance of the host, antibiotic treatment, or low pathogenic potential of the organism, may contribute to L-form production *in vivo*.

L-form conversion appears to offer the infecting bacterium a means of survival in an unfavorable host environment, since L forms can resist antibiotic therapy or host immune mechanisms that would destroy the normal bacterial form. L forms apparently can persist for years *in vivo*, because their low metabolic requirements and their very low degree of pathogenicity do not evoke an all-out defensive response on the part of the host. Furthermore, because the L form retains its capacity to revert to the parent bacterium, its transmission to new hosts remains possible. Without a doubt L formation is one of the most perfect solutions, from the point of view of the parasite, to assure microbial survival during a long term host-parasite relationship.

Recently I have had the great pleasure of visiting Dr. Chatterjee's laboratory and examining at first hand preparations of his *M. leprae* L forms. In my opinion, they are classic examples of the unstable L and transitional phases. Certain stages are almost indistinguishable from the transitional states characteristic of other bacterial species that we ourselves have studied. The fact that he has observed these forms in lepromatous tissue from untreated patients and cultured them in media free from inhibitors, is strong evidence that the L form arises spontaneously during the natural disease.

The more one thinks about the implications of L forms in leprosy, the more one is tempted to conclude that had these L forms never been discovered, their existence

would have to be postulated in order to account adequately for certain of the aspects of the pathogenesis of leprosy.

Dr. Rees. Thank you very much, Dr. Wittler. I think we should bring Dr. Reich's paper into the discussion together with that of Dr. Chatterjee. I believe it would be profitable, before throwing the paper over to general discussion, if we could hear something about bacterial genetics in relation to both these papers. I would like to ask Dr. W. Lane Barksdale, Associate Professor of Microbiology in New York University, to discuss this question.

Dr. Barksdale. I would like to outline some information that has to do with the diphtheria bacillus, information which I think offers an overall genetic pattern in agreement with the beautiful multiplication of *M. leprae* in the foot pad and with the possibility of having blob-forms of *M. leprae* that multiply in artificial media. The term blob-form is used to indicate certain mutants derived from bacillary forms of *C. diphtheriae*. These mutants vary in shape from spherical to irregular blobs. They probably are wall-less mutants. Hence we have designated them W$^-$. We are deliberately avoiding the use of the terms L-form or PPLO. So, if you will bear with me, let me remind you that *C. diphtheriae* is a gram-positive rod producing a specific toxin which has been crystalized. The diphtheria bacillus is sensitive to penicillin and to salt, and grows on ordinary media. It grows on agar with no requirement for serum. Some years ago, inadvertently and because of conditions obtaining in our defined media, we isolated from the Park-Williams 8 strain of the diphtheria bacillus a blob-form which we put away, and had no special interest in, until, later, a student was found to be carrying a blob-form that could give rise to diphtheria bacilli. We then unearthed the wall-less mutant of the Park-Williams 8 strain and found the blobs to be gram-negative; they would not grow on ordinary media; they required a complex medium containing serum. They resisted a thousand units of pencillin. They grew in the presence of 2.5 per cent sodium chloride, and yet salt is lethal for the diphtheria bacillus (Table 1).

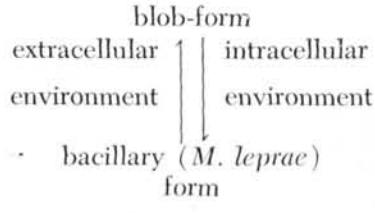
Immediately the question arises as to whether or not this was *C. diphtheriae*. This put a terrific responsibility on us because the organism failed to produce toxin, and its growth requirements were unique. How could we establish the fact that W$^-$ was related to the diphtheria bacillus? In attempting to establish a relation of W$^-$ to *C. diphtheriae* we just happened to find a mutational pattern which I think is applicable to the subject of this meeting. We tried to get a back mutation to a rod form that would produce toxin. Since we expected the mutation rate to be very low, we grew large amounts of the blob-form (W$^-$). I have said that the blob does not grow on ordinary agar; so it occurred to me that ordinary agar would be a nice selective medium on which to isolate the occasional bacillary mutants occurring in the blob population (W$^-$). We grew a W$^-$ population, spun this down, and made a pellet of more than 10^{10} organisms, seeded it out on a plate, sealed it up, and incubated it. Out of this came 7 mutant colonies, greenish in appearance, with the following properties. First and most obvious: they grew on agar. The cells were rods

TABLE 1. *Characteristics of* C. diphtheriae *and the blob form* W$^-$.

W$^-$, wall-less strain	Mutational Step I, Rate $\approx 10^{-8}$	Wall-less in broth: Broth ↓ / Agar ↑	Mutational Step II, Rate $\approx 10^{-7}$	Typical diphtheria bacilli
Resistant to 10^3 units penicillin				Sensitive to 10^0 units penicillin
Resistant to 2.5% NaCl				Sensitive to 2.5% NaCl
Osmotically sensitive		Bacilli on agar		Osmotically stable
Nontoxinogenic				Toxinogenic

when grown on agar and blobs in broth (Table 1). They were nontoxigenic, which was most disappointing. Subsequently, from a large population of these organisms, we isolated two kinds of light colored colonies: buff and white. Of these, 2 produced diphtheria toxin (300 μgm./ml.) and 5 failed to produce toxin but did produce antigens that reacted with commercial antitoxin (a preparation known to contain a variety of anticorynebacterial antibodies, other than antitoxin).

Note that (Table 1) by two mutational steps we have gone from a blob-form, W⁻ (wall-less), of *C. diphtheriae* to a standard, walled diphtheria bacillus. The rate for the first mutation is about 10^{-7}; that for the second lies between 10^{-8} and 10^{-9}. The sum of these is 10^{-15}. Thus, to prove that W⁻ is a manifestation of the genome of *C. diphtheriae* one would need to be able to select one mutation in 10^{15}; this would seem a hopeless task. It has been accomplished here, however, by: (1) using agar as a selective agent for retrieving a few greenish colony-forming mutants from a large population of the blob form; (2) selecting, from a large population of these *step 1 mutants*, rare colonies that represented a mutation to true *C. diphtheriae*. From this model, then, one can see that if an infectious agent is a blob-form it still can have the potential to give rise to genetically different end-types (mutants). If one of these is characterized by a capacity to exhibit a blob phenotype under one set of conditions and a rod phenotype under another, and if one of these conditions could be met in one part of the host and a second in another, one would see in the host tissue the easily recognized rod forms. A very discerning worker might see also the blob forms. If the rod form cannot grow, as such, outside the host and if the blob form cannot grow on ordinary laboratory media, the average microbiology laboratory would find itself confronted with an "organism that can't be cultivated." It seems to me not at all unreasonable to assume that the infective phenotype in *M. leprae* is a wall-less organism which finds the specific material needed for making its wall inside certain host cells. In the model I have drawn on the board one might substitute for purposes of discussion in the step I mutant as follows:



Dr. Chatterjee. I would like to comment on Dr. Barksdale's very interesting discussion. I assume that Dr. Barksdale is speaking of reversions from the L forms, or using his word "blobs," to the rods. I do not think this necessarily involves any problem of mutation.

Dr. Barksdale. No, I think we would be back in the 1920's if we talked about reversion in such a way. You can have a reverting phenotype such as Dr. Nickerson displayed beautifully with his fungi or the capacity to revert in this manner, which is a change in genotype (see step I mutant in diagram). W⁻ is stable. Nothing you do to this W⁻ type will give you rods. Only one cell in 10 million mutates to a step I type. This frequency is something that cannot be picked up unless you use a selective agent and literally millions of bacteria. What I have diagramed on the blackboard indicates that one cannot go from a W⁻ strain to a typical clone of *C. diphtheriae* by any route except that involving two discrete mutational steps. This also means that the genome of the W⁻ strain contains the potential for such mutational events. Therefore, the steps that led to the creation of the W⁻ strain from the PW8 strain must have been point mutations or suppressor mutations, not mutations resulting from a deletion of part of the genome. It seems to me, therefore, that you should try to get some rods from the blob-forms you have described today even if those rods are not identical with the rods that are obtained in foot pads. The potential for a multiplicity of expression, the *genomic* potential of the leprosy bacillus (provided it really is not an intracellular parasite),

should be just as great as the genetic potential of any bacterium.

Dr. Chatterjee. Would you give a little bit more characterization of the blobs. I am interested to know exactly what you mean by the blobs.

Dr. Barksdale. We grow them in roller tubes. They are difficult to tell from coagulated serum, a fact with which you must be well acquainted if you have prepared such things from a complex medium for microscopic examination. They have the capacity to grow and we can get an optical density of 1.2 finally; this is equivalent to something like 5×10^9 bacteria. From these we can make good pellets.

Dr. Chatterjee. How do they look morphologically, i.e., as compared with the bacillus?

Dr. Barksdale. If you don't fix them with methanol before staining them with the gram stain, you just get a weft of gram-negativity. These are, of course, gram-negative, as I indicated on the blackboard.

Dr. Rees. Thank you, Dr. Barksdale. These papers are now open for general discussion.

Dr. Shepard. I want to point out that there is no question any more that PPLO and L forms exist, and I do not think it meets the criticism of this type of work in leprosy to describe all of the morphologic changes that occur with other organisms. The difficulty with this work is that there are so many interpretations of the results. To present blobs and spheres in kodachrome pictures it not necessarily presenting data on *M. leprae*. For example, one needs to consider with a critical mind some of the other possible interpretations. The forms presented could all be artifacts, for example; we have had no demonstration that they are not artifacts. We know that if we put *M. leprae* into a tube, we have to put in lots of tissue also. But what happens to the tissue in the tube? We need supporting evidence all along the line that the forms we are shown are actually *M. leprae*. Especially in the case of unusual nonbacillary morphology, we need more supporting evidence, not less.

Dr. Rees. I will now call on Dr. John H. Hanks, Chief of the Johns Hopkins-Leonard Wood Memorial Leprosy Research Laboratory at Baltimore.

Dr. Hanks. I am not quite sure the construction of our program has been quite ideal. We may have covered no less than 50 questions which deserve far more thoughtful and complete consideration. First, I might emphasize that growth rates of the protoplastic portion of microbes often exceed the rates of cell wall formation. It is a classic observation that, during the normal growth cycle, young, vigorously growing cells are much less hardy than the mature cells harvested while growth is being completed or is stationary. Dr. Moulder described the soft-walled forms of psittacine bacteria, which grow at a tremendous rate but are not transmissible. Later, as wall formation matures, they become toxic and transmissible. This exaggeration of the normal phenomenon seems to be shared by *M. leprae*. According to the descriptions of Chatterjee and Reich, *M. leprae* can initiate the soft-walled forms of growth *in vitro*, but success in converting cells to complete bacilli is small. Because of the great variety of forms seen, and the inability to define their numbers or viability, it is obvious that much thoughtful work is required.

A second point is that we all have focused on the rugged-walled, acid-fast forms of the mycobacteria. The importance of spheroplasts and weak-walled masses has escaped attention. We grind pellicles and tissues, we treat with enzymes, we wash in water, and we look at dried preparations for acid-fast rods. No one will quarrel with the view that they are the infectious elements in the transmission experiments of Drs. Shepard and Rees. It seems, however, that the time has come when our procedures must be more gentle and when the viability and significance of the delicate forms of mycobacteria must be studied.