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Morphological Changes of *Mycobacterium lepraemurium* Grown in Cultures of Mouse Peritoneal Macrophages[1]

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Studies were made on morphological changes of *Mycobacterium lepraemurium* grown in cultures of mouse peritoneal macrophages. Two types of nonsolid or irregularly stained *M. lepraemurium* were observed. One type occurred in the growth phase of the organisms during the stage of preparation for bacillary multiplication. The nonsolid bacilli appeared as elongated organisms having pointed ends, isolated acid-fast dots, or faintly stained areas at the ends of the bacilli. It is possible that this irregularity in staining is due to a very gradual, versus an instantaneous, acquisition of acid-fast material during bacillary multiplication and maturation. Solid forms were again observed upon maturation. Nonsolid bacilli were also observed in macrophage cultures infected with autoclave-killed *M. lepraemurium*. Under these conditions there was an emergence of organisms which showed irregularly stained areas and various forms of deformity unaccompanied by elongation or multiplication. These irregularities were most probably due to the destructive process of digestion of bacillary protoplasm. The present study does not support the current hypothesis that all nonsolid acid-fast organisms are nonviable.

Since standard measurements of viability are not applicable to organisms which are noncultivable in vitro, the morphological appearance of human leprosy bacilli has been used for some time to surmise whether organisms are alive, dead, or dying. The idea that bacillary viability can be assessed by the morphological appearance of organisms originated from studies of Rees and associates on the growth of *Escherichia coli* and *Mycobacterium lepraemurium* in vitro, on the growth of *M. tuberculosis*, *M. lepraemurium*, and *M. leprae* in mice, and on the morphological changes of *E. coli*, *M. lepraemurium*, and *M. leprae* as observed with the electron microscope (13, 14, 16, 17). Observations made during these studies led them to claim that all solidly stained organisms were alive and all nonsolidly stained ones were degenerate or dead.

Our present study on morphological changes of *M. lepraemurium* grown in macrophage cultures does not support the contention that all nonsolid organisms are dead. On the contrary large numbers of irregularly stained or nonsolid organisms are observed during the growth of *M. lepraemurium*.

MATERIALS AND METHODS

Techniques for the cultivation of *M. lepraemurium* in cultures of mouse peritoneal macrophages have been reported previously (6, 9). Briefly, peritoneal exudate was harvested from female ex-breeders of the general purpose strain of National Institutes of Health Swiss white mice. For regular bacillary growth, macrophages were maintained in a basal medium consisting of 40% horse serum, 50% medium NCTC 109, and 10% of a 1:5 dilution of bovine embryo extract. This was supplemented with liver extract L fraction, 1 mg/ml, and ferric nitrate, 1 μg/ml, for faster bacillary growth. Cultures were kept at 37 C in an atmosphere of 5% CO_2-air mixture.

Organisms for the first experiments were obtained from a mouse which had been infected with the Hawaiian strain of *M. lepraemurium* 89 days previously. Short forms of these organisms proved convenient for morphological studies during growth, since observations of bacillary elongation were more easily made. Organisms used for the second series of experiments were obtained from the 16th subculture

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of a strain of *M. lepraemurium* serially transferred in macrophage cultures. These bacilli, though moderately long in form, offered an opportunity for the study of morphological changes at faster rates of growth. The third experimental series was performed with autoclave-killed bacilli obtained from the 10th subculture of another strain of *M. lepraemurium* serially transferred in macrophage cultures. Morphological changes observed in these experiments represent those which occur after the death phase of bacterial growth.

Infection of macrophages was made by introducing a suitable number of organisms into the culture on the day after establishment of the monolayer. Leighton tubes containing cover slips (8 by 22 mm) were used. Cover slips were examined on the day after infection and at weekly intervals thereafter for a period of 7 to 12 weeks. The slips were fixed in Zenker's stock solution and stained with Ziehl-Neelsen acid-fast stain followed by hematoxylin stain (Harris). Methods for enumeration of intracellular bacilli have been reported previously (9). Measurement of the length of a bacillus was made with a scale in the ocular, each division being 0.55 μm. The length of the bacillus was recorded as the average for 100 bacilli, based on the following schemes: 1 to 2, 3 to 4, 5 to 6, 7 to 8, 9 to 10, etc. For example, if the percentages are 60, 30, 6, and 4, respectively, then the length of the bacillus will be $[(60 \times 1.5) + (30 \times 3.5) + (6 \times 5.5) + (4 \times 7.5)]/100 \times 0.55 = 1.4$ μm.

RESULTS

The growth pattern and morphological changes of the short form of *M. lepraemurium* in macrophage cultures are shown in Fig. 1. Since the organisms were obtained directly from an infected mouse, a long lag phase was needed for adaptation into the tissue culture environment. Bacillary multiplication was first observed 5 weeks after infection and became marked after 10 weeks of cultivation. A 26-fold increase in the number of organisms was observed at the end of 12 weeks.

Irregularly stained or nonsolid forms appeared shortly after cultivation. Various forms of irregularity were observed; i.e., organisms with an acid-fast dot at one or both ends of the bacilli, organisms with pointed ends, short or long chains of acid-fast dots, segmented bacilli, organisms with faintly stained segments, or organisms with various forms of curvature, as shown diagrammatically in Fig. 2. The proportion of all irregular forms increased during cultivation, especially those with terminal dots or with areas of faint staining. The total of nonsolid organisms increased from 31% in the beginning to 66 to 77% throughout the period of observation (Fig. 1 and 2).

As seen in Fig. 1, marked elongation was observed during the entire cultivation period and long before the appearance of bacillary multiplication.

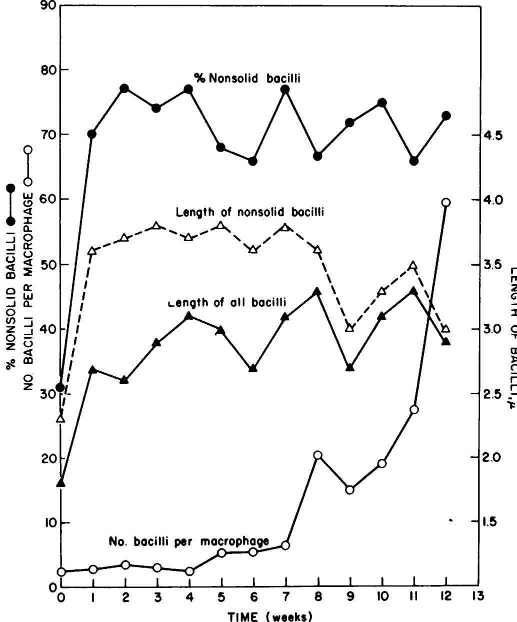

FIG. 1. *Growth pattern of M. lepraemurium in macrophage cultures. The organisms were obtained from a mouse of 89-days infection and were mostly short forms. Note the marked increase in the percentage of nonsolid bacilli long before the appearance of bacillary multiplication.*

The average length of all organisms was 1.8 μm the day after infection. This average increased to 2.7 μm in one week and fluctuated between 2.6 and 3.3 μm thereafter. The average length of the nonsolid bacilli increased from 2.3 to 3.6 μm in 1 week and remained at 3.6 to 3.8 μm thereafter before a slight decrease in the length at the ninth week of cultivation.

The possibility that nonsolid organisms were actually growing was suggested by their elongation. The bacillary population used in this experiment contained a large portion of short forms; thus an opportunity was offered to assess their viability in cultures. This was evaluated, since very short, uniform bacilli, having a length no greater than 3 to 4 times their diameter, have been considered by Waters and Rees (21, and *personal communication*) as degenerate forms or dead bacilli. Figure 3 shows changes in proportion of organisms of various length during the growth phase of *M. lepraemurium*. The proportion of short organisms (1–2 and 3–4 micrometer scales) decreased as the longer ones (5 to 6 and 7 to 8 scales) increased throughout the experiment. Very short organisms, 1 to 2 scales or 0.55 to 1.1 μm in length, dropped from 30 to 5% in 1 week. Since dead acid-fast organisms can remain in cell

	SOLID	ONE DOT	TWO DOTS	POINTED ENDS	CHAIN 2 DOTS	CHAIN >2 DOTS	SEGMENTED	IRREGULAR STAINING	CURVED
TIME									
1 day	69	8		5			3	10	5
1 week	30	25	10	4	2		11	12	6
2 weeks	23	8	5	9		1	15	33	6
3 weeks	26	16	2	1			11	37	7
4 weeks	23	5	5	1			17	46	3
5 weeks	32	3	2	1		2	14	43	3
6 weeks	34	7	1	1	1	1	10	40	5
7 weeks	23	8	3	1		3	15	40	7
8 weeks	33	11	1		2		13	37	3
9 weeks	28	11	3		5	5	21	21	6
10 weeks	25	9	5				25	31	5
11 weeks	34	7	4	1	4		17	27	6
12 weeks	27	4	1	1	4	7	13	34	9

FIG. 2. *Morphological changes of M. lepraemurium in macrophage cultures. Changes are diagrammatically presented; the actual appearance of these organisms is shown in the micrographs in Fig. 4. Values represent percentages of the total of 200 organisms counted. Note the increase of various forms of nonsolid organisms with a simultaneous decrease of the solid ones.*

culture for a long time before they are completely digested, the rapid disappearance of very short organisms with the simultaneous increase of longer ones could only be interpreted as evidence of elongation of the short bacilli. Furthermore, the fact that the percentage of phagocytosis remained more or less unchanged throughout the experiment would indicate that there had been no actual disappearance of the very short organisms; otherwise, the percentage of phagocytosis would have dropped markedly when the number of short organisms was drastically reduced.

More direct evidence of elongation of the very short solid organisms was observed microscopically. The deeply stained short bacilli appeared to have developed faintly stained segments extending from both ends of the bacilli (Fig. 4). Possibly, with the development of more acid-fast material in the new segments, a solid, long bacillus eventually emerged.

Morphological changes of *M. lepraemurium* which had been maintained in serially transferred cultures were studied next, Fig. 5. Like the short organisms in above experiments, these longer organisms showed the emergence of non-

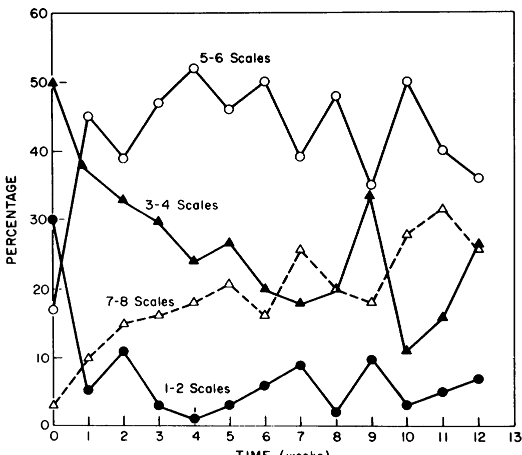

FIG. 3. *Changes in the length of M. lepraemurium in macrophage cultures. Note the decrease in the percentage of organisms of 1–2 and 3–4 micrometer scales and the simultaneous increase of those of 5–6 and 7–8 scales. Each scale equals 0.55 μm.*

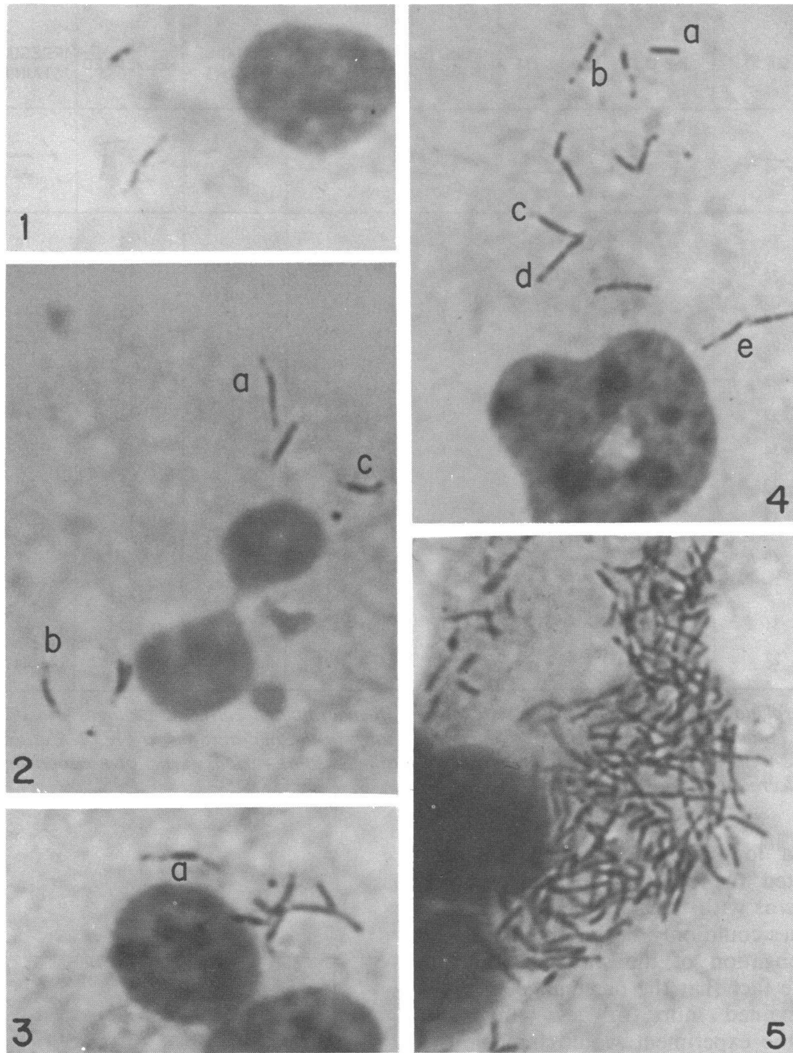

FIG. 4. *Growth of M. lepraemurium in macrophage cultures. Organisms are stained with Ziehl-Neelsen acid-fast stain. Macrophages are stained with hematoxylin. The color in the solid portions of bacilli is deep red, whereas the faintly stained portions are pink. The nuclei of the macrophages are blue in color.* × *2,000. Part 1. One-week-old culture. In the center, there are two short solid bacilli in a row with faintly stained areas extending from the outer ends. Part 2. One-week-old culture (a) and (b). Moderately long, solid bacilli, each having faintly stained. extension on both ends. (c) Curved bacillus with faintly stained area at one end. Part 3. Four-week-old culture (a) Bacillus with a short, solid center having long faintly stained rods extending from both ends. Several long, solid organisms are seen in the upper right. Part 4. Four-week-old culture. (a) Solid bacillus. (b) Two nonsolid organisms, each containing two or three dots. (c) Bacillus showing faintly stained, pointed ends. (d) Segmented bacillus. (e) Two bacilli in which the faintly stained areas show more acid fastness than those seen in Part 1 and 2, suggesting an increase in acid fastness on maturation of the bacilli. Part 5. Twelve-week-old culture. Most organisms exist as solid forms.*

solid bacilli which appeared to be viable and growing. These organisms grew faster in macrophage cultures than those obtained directly from the mouse. In comparison with the previous experiments (Fig. 1), a shorter lag phase before bacillary multiplication (1 week rather than 4) was observed. The organisms showed a 55-fold increase in number in 6 weeks and, although already moderately long, showed an overall 0.9-μm increase in length in 3 weeks. During the

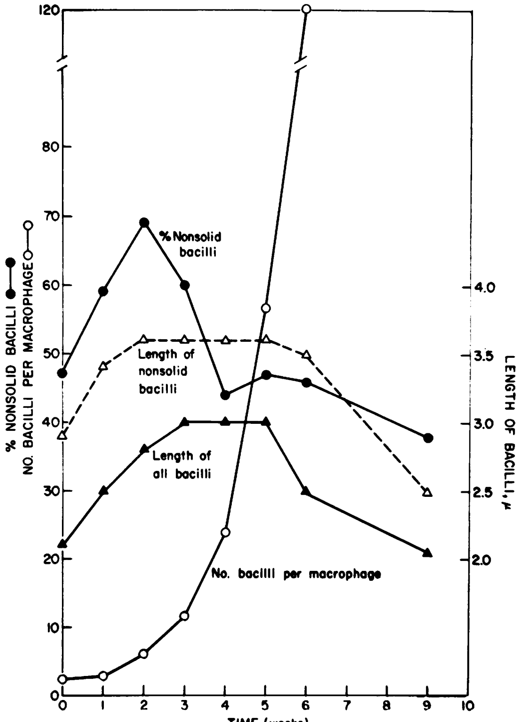

FIG. 5. *Growth pattern of M. lepraemurium in macrophage cultures. The organisms were obtained from serially transferred cultures. A faster growth rate of M. lepraemurium was observed in this experiment than that shown in Fig. 1. Note the marked increase in the percentage of nonsolid organisms in the first 2 weeks, followed by a rapid fall when the logarithmic phase emerged.*

first 2 weeks of observation, nonsolid organisms increased in proportion from 47 to 69% and increased in length by 0.7 μm. As the total number of bacilli increased logarithmically between 2 and 4 weeks, the percentage of nonsolid organisms decreased rapidly to the original level. A rapid turnover of nonsolid into solid forms during the logarithmic phase was suggested.

When autoclave-killed *M. lepraemurium* were added to macrophage cultures, an increase in proportion of nonsolid forms was observed but no elongation of these forms could be measured. In addition, although the nonsolid bacilli increased from 16 to 66% during the 72-day period, no increase in the number of organisms was observed, Fig. 6. Curved bacilli and irregularly stained organisms (those with faintly stained areas) were most prominent, Fig. 7. Segmented bacilli increased slightly but chain form organisms or those with terminal dots showed no marked change in number (Fig. 8).

Based on these findings, it may be concluded that nonsolid bacilli can be observed in both the growth phase and during deterioration of *M. lepraemurium* in macrophage cultures. Elongation of nonsolid organisms is observed only in the growth phase. The growth of very short uniform (solid) bacilli is also correlated with elongation.

DISCUSSION

The present study clearly suggests that irregularly stained *M. lepraemurium* which appear in the growth phase may not be dead or degenerate but may indeed be growing. The growth seems to start as an elongation of end portions which are initially filled with scantly staining acid-fast material. Acid fastness increases with maturation until a solid organism eventually forms. It is likely that two types of nonsolid forms exist with *M. lepraemurium:* those occurring in the adaptive stage of preparation for bacillary growth when acid fastness is being developed, and those occurring during or after the death phase as a result of lytic destruction of dead bacillary protoplasm. Emergence of the young, nonsolid forms during the lag and logarithmic phases of the growth cycle is accompanied by definite signs of elongation which do not appear with the emergence of the nonsolid forms

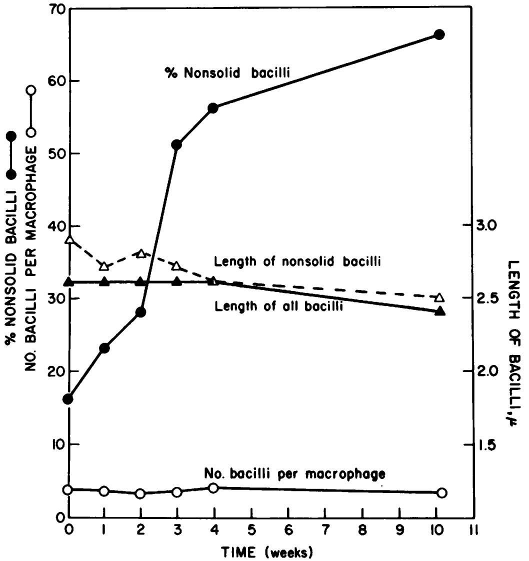

FIG. 6. *Changes of killed M. lepraemurium macrophage cultures. The organisms were obtained from serially transferred cultures but were autoclaved before inoculation. Note the marked increase in the percentage of nonsolid organisms without a simultaneous increase in the number or length of the bacilli.*

KILLED BACILLI

TIME	SOLID	ONE DOT	TWO DOTS	POINTED ENDS	CHAIN 2 DOTS	CHAIN >2 DOTS	SEGMENTED	IRREGULAR STAINING	CURVED
1 day	84	2		1	1	1	1	3	7
7 days	77	5		2		1	7	6	2
14 days	72	5					6	7	10
21 days	49	7		1		2	5	21	15
28 days	44	5					5	23	23
72 days	34	5		2			1	29	29

FIG. 7. *Morphological changes of killed M. lepraemurium in macrophage cultures. Values represent the percentages of the total 200 organisms counted. There was a marked decrease of solid organisms with a simultaneous increase of nonsolid bacilli, particularly the irregular-staining and curved ones.*

during the death phase. Clinical support for this contention comes from the work of Ridley and Ridley (18), who recently reported that the club forms, i.e., the irregularly stained forms having metachromatic granular inclusions, were actually associated in some way with the early phase of regeneration of *M. leprae* in some patients who relapse.

Rees and associates (16) using *E. coli* and *M. lepraemurium* suspended for various lengths of time in phosphate buffer at 37 C, demonstrated a correlation between the solid appearance of the organisms under electron microscopy and their viability and have advanced the proposition that all solid organisms are viable and all nonsolid or irregularly stained organisms are degenerate or dead. Support for this idea is gained by their later experiments on murine leprosy treated with isoniazid, on *M. tuberculosis* infection in mice treated with isonizaid-pyrazinamide, and on mouse foot pads infected with *M. leprae* that were obtained from leprosy patients after treatment with 4,4′-diaminodiphenyl sulfone (DDS; 13, 16, 17). These experiments led these authors to surmise that bacillary viability assessed by morphological appearance could be applied to human leprosy bacilli.

The data presented in the present report demand that a more critical view must be taken of the conclusion that all nonsolids are dead. An examination of the data given by Rees and associates reveals that (i) their in vitro tests were performed in phosphate buffer at 37 C for 28 to 56 days or longer, and (ii) the organisms used for their in vivo tests were obtained from murine leprosy treated with isoniazid for 8 to 12 months (16), from *M. tuberculosis* infection in mice treated with isoniazid-pyrazinamide for 8 months (17), and from leprosy patients treated with DDS for 12 months or more (13). It is known that phosphate buffer does not support growth and that the infectiousness of *M. lepraemurium* declines rapidly at 37 C (10). Organisms maintained under such conditions would most likely be engaged in the death phase of bacterial growth. Surely the organisms utilized for in vivo tests were very likely dead or at best in poor condition. What was proved essentially was that dying or dead nonsolid organisms were nonviable. It should be noted that nonsolid organisms obtained from the growth phase of these organisms were not tested. The possibility that viable nonsolid organisms might exist was not examined.

Comparative studies have been made by Rees and Valentine (14) to support the feasibility of using light microscopy for the determination of bacillary viability. First, a diagrammatic sketch was made of Ziehl-Neelsen stained organisms as observed under light microscopy. The same preparation was subjected to electron microscopy, and photomicrographs were obtained for comparison with the diagram. A match of corresponding figures led to claims that the electron-microscopic appearance could be reproduced by Ziehl-Neelsen staining under light microscopy, and that the irregularly stained organisms observed under light microscopy were the degenerate forms observed under electron microscopy. Unfortunately, the experimental data do not support the conclusion for the following reasons. First, determination of bacillary viability by using morphology as a basis would depend chiefly on the condition of the internal structures of organisms such as cell

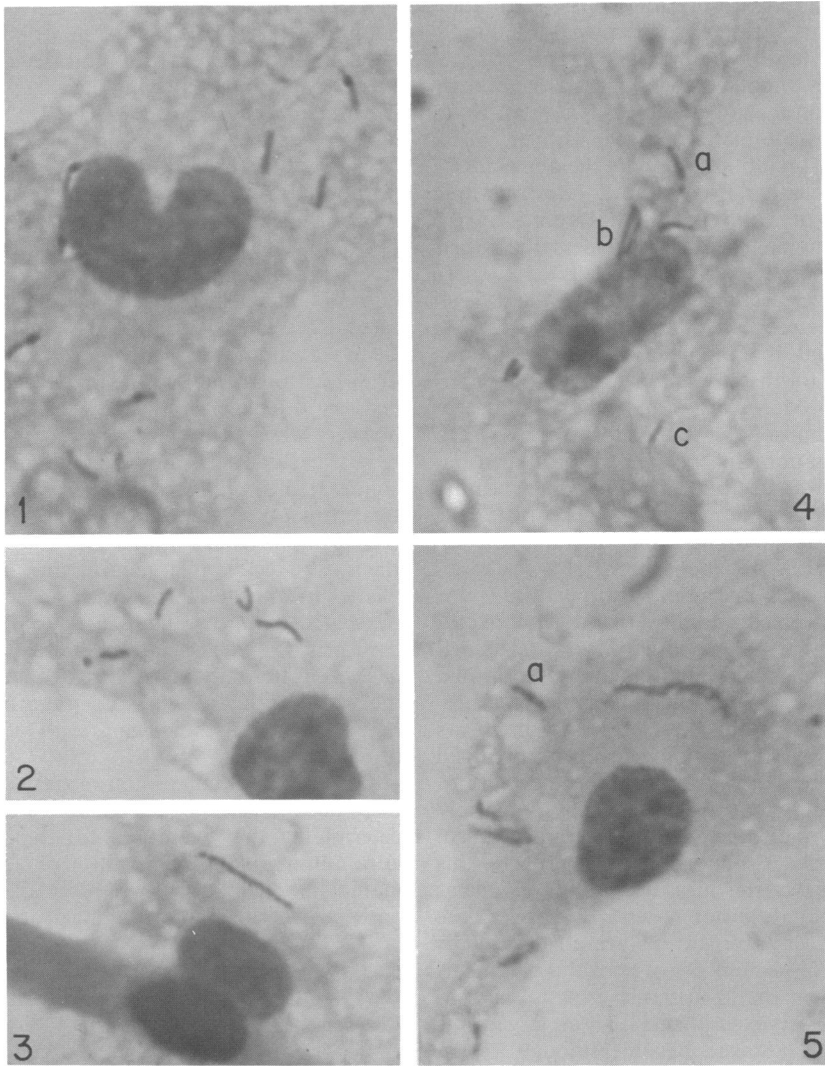

FIG. 8. *Morphological changes of autoclave-killed M. lepraemurium in macrophage cultures. Organisms are stained with Ziehl-Neelsen acid-fast stain. Macrophages are stained with hematoxylin. The color of bacilli is deep red. The nuclei of macrophages are blue in color.* × *2,000. Part 1. One-day-old culture. All organisms are in solid form. Four bacilli contain one or two large, metachromatic (deep blue) granular inclusions. Part 2. One-week-old culture. Two curved bacilli in the upper right. Part 3. Two-week-old culture. One long bacillus with cytoplasm displaying varying degrees of acid fastness. Part 4. Four-week-old culture. (a) Solid bacillus with one dot at each end. (b) Two bacilli: one in solid form, and one showing thin center. (c) Faintly stained bacillus with pointed lower end. Part 5. Seventy two-day-old culture. (a) Solid bacillus containing a metachromatic granule is still present. Other bacilli reveal curvatures or faintly stained areas.*

membrane, nuclear and mesosomal patterns, etc. Since ultrathin sections were not used in their electron microscopy, it is difficult to judge whether the internal anatomy of the organisms, many of which contained solid portions, was intact or degenerate. Second, discrepancies appear to have been encountered in identifying the same organisms by light and electron microscopy. The light microscopic sketch of *M. leprae* by Rees and Valentine (14) does not correspond to its electron photomicrograph, nor to the light microscopic sketch cited by Rees and Waters (17), which is supposed to be a duplication of the same picture.

Rees and Valentine (15) also asserted that the heating process used in the standard Ziehl-Neel-

sen staining caused spreading or redistribution of cytoplasmic contents of nonsolid bacilli, resulting in a falsely uniform appearance. Consequently, they modified the standard method by utilizing a lower temperature (37 C) for 6 to 15 hr. However, the pictures shown in their earlier publication (14) do not support the adequacy of such a modification. For example, the cytoplasmic content of nonsolid bacilli, according to the diagrammatic sketches, is much less than their corresponding electron photomicrographs. These organisms were stained in steaming Ziehl-Neelsen. The staining might have been even less thorough had the modified staining technique been used. In the present study, the standard staining procedure was employed. A large proportion of nonsolidly stained organisms was a usual observation. In contrast to the redistribution hypothesis of Rees and Valentine, heating has been found necessary to accentuate the cytoplasmic contents in the staining of *M. lepraemurium* in this laboratory.

Recent studies on mouse foot pads infected with *M. leprae* have led Shepard and McRae (19) to conclude that the proposal of Rees and associates, concerning the viability status of nonsolid leprosy bacilli, was confirmed by direct measurements of infectivity in mice. Their measurements were made by inoculating organisms obtained at various stages of development into new groups of mice. They found that the capacity to multiply was directly related to the ability of an organism to stain solidly. Examined more rigorously, this finding supports the idea that solid organisms are viable but does not necessarily support the idea that nonsolid organisms are all dead. It is notable that large numbers of nonsolid organisms accumulated in the logarithmic growth phase in these foot pad experiments. These organisms were regarded as dead bacilli, although a progressive death of organisms would ordinarily be associated with a death phase rather than a logarithmic growth phase. It seems more logical to suppose that these organisms were the juvenile forms of *M. leprae*, similar to those observed in young cultures of *M. tuberculosis* (11) and in the lag and logarithmic phase of *M. lepraemurium* described above. These young organisms might not have been dead despite their failure to grow in foot pads upon subinoculation. Since multiplication of *M. leprae* in foot pads appears to occur only with solid *M. leprae*, and then to a limited degree, one is doubtful whether this system, with its apparently growth-limiting nature, is suitable for judging the viability of any nonsolid *M. leprae*. Such organisms might display an unsuspected degree of liveliness if inoculated into a more susceptible host, such as the human body.

In summary, evidence has been presented to support a belief that not all nonsolid organisms should be considered dead, and the suggestion is made that not all morphologically intact orgaisms should be assumed viable. The latter corollary is supported by studies of Shepard and McRae (19), who reported that a constant proportion (10%) of solid bacilli in mouse foot pads was not infectious and was most probably dead, remaining solid in appearance owing to a very slow protoplasmic deterioration. This phenomenon may also have been observed in the case of the clinical trial of the antileprosy drug B.663, a rimino derivative of phenazine. This drug was found by Browne and Hogerzeil (3) to be very effective in the treatment of leprosy; however, resistance of *M. leprae* was thought to have developed because of the recurrence of solidly stained organisms after 1 year of continued treatment (4). Concurrently, B.663 was found by one of the present authors (Y. T. Chang) to be the most effective treatment in chemotherapeutic studies in murine leprosy with no emergence of resistance in mice after 816 days of continued treatment (5, 7, 8). Up to the present time, B.663 has been tried in leprosy patients throughout the world (1, 2, 12, 20), and there has been no report of any cases showing signs of emergence of resistance.

The important conclusion of this report is that there is still a lack of dependable techniques for determining the viability of *M. leprae* both in vitro and in vivo. Great caution should be taken in judging the probable viability of *M. leprae*, both in experimental and clinical investigations.

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