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Electron Microscopic Observations of Intracytoplasmic Inclusions in Human and Murine Leprosy Bacilli[1]

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The intracytoplasmic granules of mycobacteria were first demonstrated as discrete, dense electron-scattering bodies by Lembke and Ruska (9) and by Wessel (14). They distinguished *Granula* (300–350 mμ in diameter) and *Mikrogranula* (20–30 mμ in diameter). Imaeda and Convit (3) found moderately dense, presumably polyphosphate bodies in the cytoplasm of *Mycobacterium leprae*. They suggested that since neither the electron-dense granules nor the intracytoplasmic membrane systems were found in degenerating *M. leprae* cells, these two components may represent indicators of the state of activity of the bacilli. Whitehouse, *et al.* (15) found larger bodies (mean diameter approximately 175 mμ, range about 120–300 mμ) in *M. lepraemurium*. These larger bodies were of moderate electron density and were seen in sections to be finely granular, homogeneous masses of material not bordered by membranes.

However, much still remains unknown regarding the fundamental properties of these intracytoplasmic inclusions in human and murine leprosy bacilli. The present paper is a report of electron microscopic observations on the intracytoplasmic inclusions of *M. leprae* in human lepromata and *M. lepraemurium* in murine lepromata.

MATERIALS AND METHODS

Human lepromata. Tissue specimens from newly diagnosed patients with advanced lepromatous leprosy were examined by skin biopsy.

Murine lepromata. *M. lepraemurium*, Hawaiian strain, have been maintained in mice for more than 15 years in our Institute by serial transmission at five- to six-month intervals. Female mice (18–20 g) of the ddY strain were given intraperitoneal injections of 0.25 ml (containing approximately 10^6 acid-fast bacilli) of a partially purified suspension of *M. lepraemurium* prepared from the homogenized liver of a mouse injected four to six months previously. In the present studies, the mice were sacrificed six months after injection.

Preparation of tissues for electron microscopy. The human lepromata and murine lepromata were immediately cut into 1 mm cubes and fixed by immersion in osmium tetroxide buffered to pH 6.4–6.6, according to the technique of Kellenberger, *et al.* (5). The tissues were dehydrated in graded alcohols and embedded in methacrylate resins by means of ultraviolet polymerization (8, 13). The ultrathin sections were processed serially on a LKB-ultratome and picked up on Formvar-covered grids. The sections were not stained. The material was examined on a JEOL-100C and/or a HITACHI-500 electron microscope operated at 50–75 kV.

RESULTS

Intracytoplasmic inclusions were observed in the bacillary cells of *M. leprae* and *M. lepraemurium*. The inclusions were mostly homogeneous and spherical, and were not uniformly distributed throughout the cells (Figs. 1–6). The inclusions in the cells of *M. leprae* had an average diameter of about 0.15 μ with a range of about 0.06 μ to 0.25 μ. In *M. lepraemurium*, they had an average diameter of about 0.25 μ with a range of about 0.08 μ to 0.5 μ.

The large, diffuse inclusions observed in the cytoplasm of *M. lepraemurium* as shown in Figures 5 and 6 were not seen in *M. leprae*. The electron-dense granular inclusions in *M. leprae* as shown in Figure 2 were not

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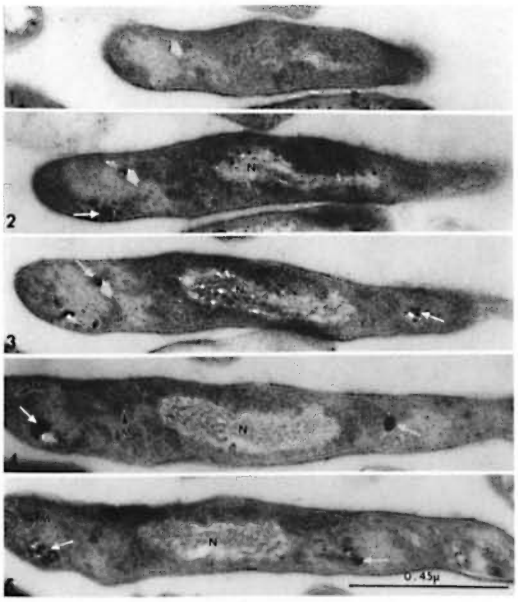

FIG. 1. Longitudinal serial thin sections of *M. leprae.* Large arrows in photos 1, 2, and 3 show the inclusions; small arrows in photos 2, 3, 4, and 5 show the electron-dense, small particles or granules. M = mesosome; N = nuclear apparatus.

observed at all frequently. They did not appear to be delimited by any membranous structure and apparently had no internal structure in general (Figs. 1–6). However, some electron micrographs of *M. lepraemurium* showed fragmentary membranous structures near the inclusion (Fig. 5). It was assumed that these inclusions were composed of osmiophilic matter which was not deformed in the process of preparation for electron microscopic examination, since these inclusion structures did not show any alterations caused by electron bombardment.

In the cytoplasm surrounding these inclusions, there were some small, electron-dense particles or granules near the cytoplasmic membrane (Figs. 1, 5, and 6). Their average diameters were 0.0135 μ in *M. leprae* and 0.0125 μ in *M. lepraemurium.* Mesosomes and nuclear apparatus were often seen adjacent to the inclusions (Figs. 1 and 4). Additionally, other intracytoplasmic membrane systems, different from the mesosomes in their morphological features, were observed in the bacillary cells (Fig. 4).

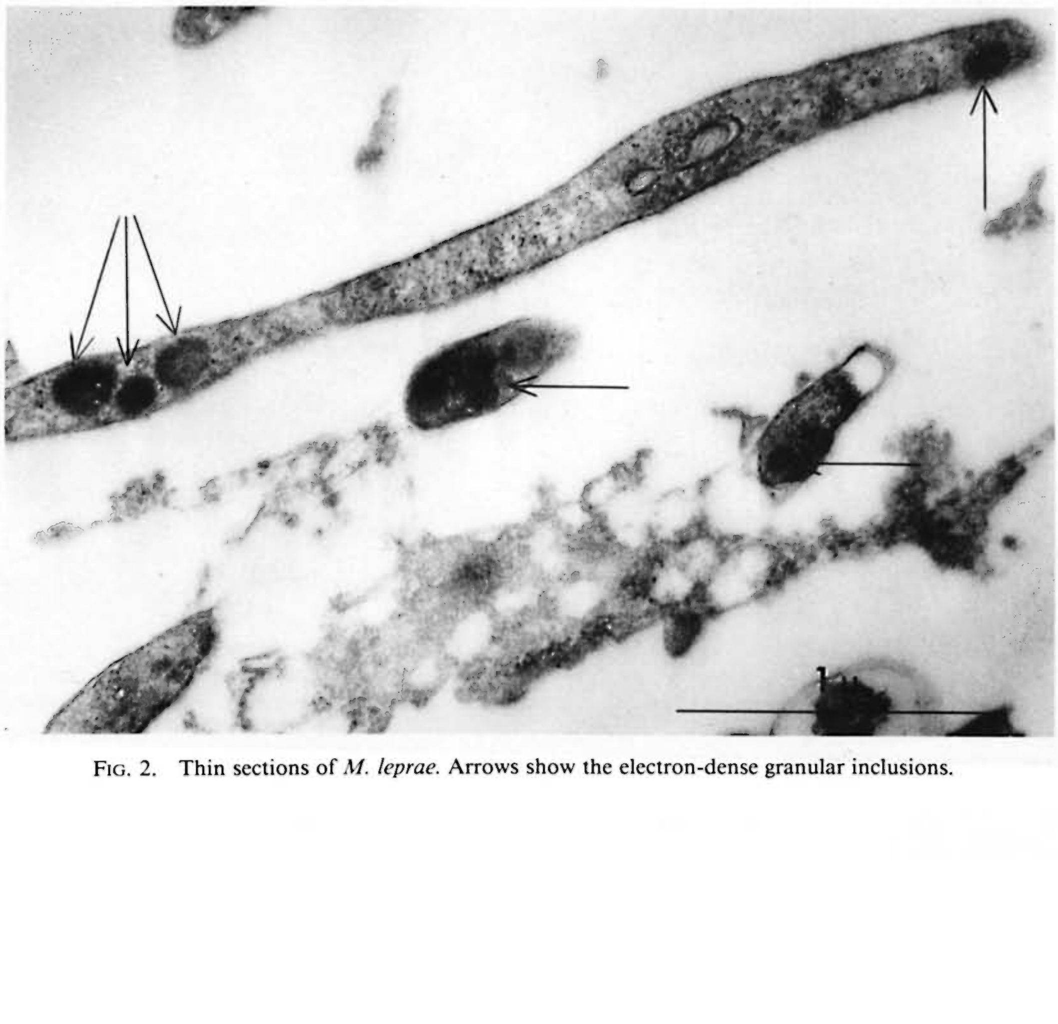

FIG. 2. Thin sections of *M. leprae.* Arrows show the electron-dense granular inclusions.

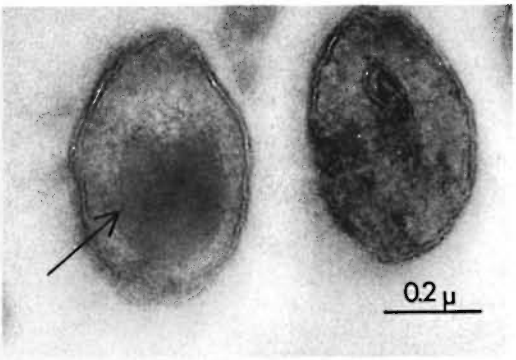

FIG. 3. Thin cross-sections of *M. leprae*. Arrow shows the inclusion. This inclusion is not delimited by any membranous structure and has no internal structure.

DISCUSSION

Intracytoplasmic inclusions or inclusion-bodies and granules or granular-substances have been reported in several species of mycobacteria. Mudd, *et al.* ([10]) stated that small, electron-scattering granules were discernible in the cells of *M. thamnopheos* and *M. tuberculosis* var. *bovis*, BCG strain. Toda, *et al.* ([12]) showed that the protoplast of *M.*

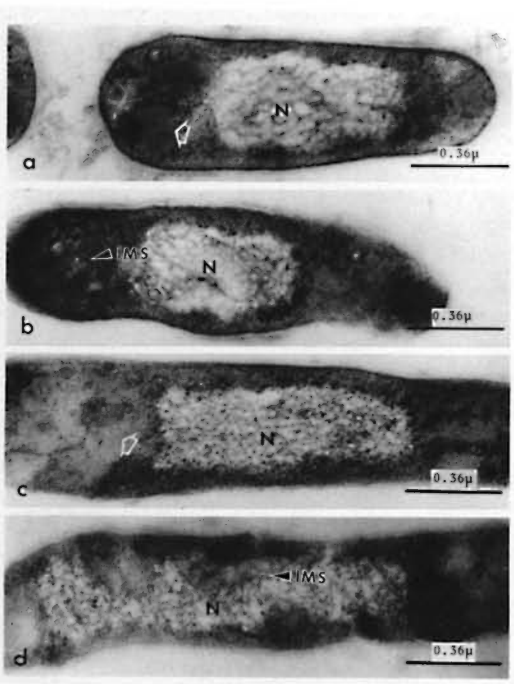

FIG. 4. Longitudinal thin sections of *M. lepraemurium*. The inclusion is seen adjacent to the nuclear apparatus (arrows in photos a and c). IMS = intracytoplasmic membrane systems; N = nuclear apparatus.

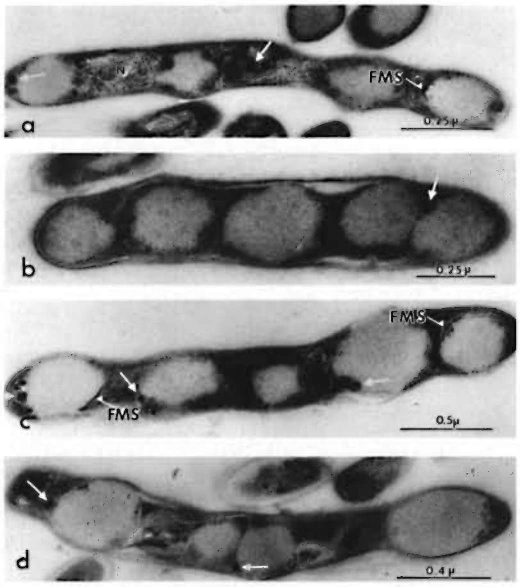

FIG. 5. Longitudinal thin sections of *M. lepraemurium*. Large diffuse inclusions are observed in the bacillary cytoplasm. Arrows show the electron-dense, small particles or granules. FMS = fragmentary feature of membranous structure; N = nuclear apparatus.

tuberculosis var. *bovis*, BCG strain, included dense, electron-scattering granules. Koike and Takeya ([7]) pointed out polyphosphate granules in the cells of *M. avium*, Jucho strain.

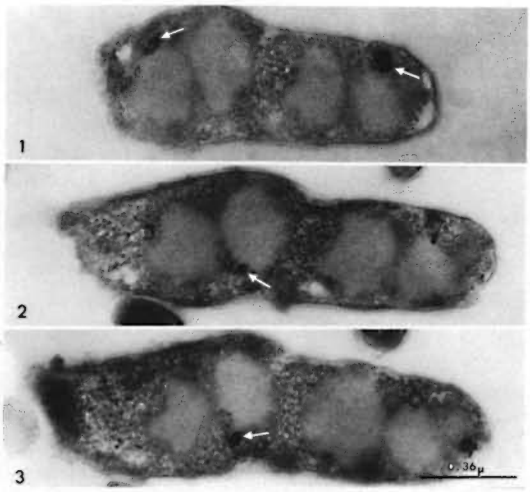

FIG. 6. Longitudinal serial thin sections of *M. lepraemurium*. Large diffuse inclusions are not delimited by any membranous structure and have no internal structure. Arrows show the electron-dense, small particles or granules.

In electron microscopic studies of *M. leprae*, Imaeda and Convit ([3]) demonstrated moderately dense bodies in the bacterial cytoplasm. Edwards ([2]) described granules of variable electron density in the cells of *M. leprae*. Whitehouse, *et al.* ([15]) reported that small, electron-dense particles and larger bodies, which were not as electron-dense as the smaller particles, were found in cells of *M. lepraemurium* isolated from mouse lepromata.

Intracytoplasmic granules and/or inclusions, known as metachromatic, Babès-Ernst or volutin granules, are present in abundance in diphtheria, plague, and mycobacterial species, and in a number of other bacteria ([11]). In bacteria of medical interest, lipid inclusions, granules of glycogen and starch, and metachromatic granules are found as intracytoplasmic granules and/or inclusions. Sulfur granules and protein crystals occur in some bacteria. Vacuoles containing gas or liquids have also been demonstrated in bacteria. Histo- and cyto-chemical observations and chemical analyses on material extracted from bacteria indicate that the main constituent of the metachromatic granules and/or inclusions is polymetaphosphate. Lipid granules seem to be the most common type of cytoplasmic inclusion of a lipid nature. Chemical analyses indicate that these granules contain poly-β-hydroxybutyric acid and some other lipid ([6]).

The intracytoplasmic granules in tubercle bacilli reduce tetrazolium salt and are stained with red formazan (reduced tetrazolium) ([4]). Mudd, *et al.* ([10]) interpreted these findings as indicating that these centers of active oxidation-reduction in mycobacteria are compatible with a revised definition of bacterial-mitochondria.

Imaeda and Convit ([3]) observed that the morphological appearance of the homogeneous, dense substance, namely inclusion, in the bacillary cells of *M. leprae* showed close similarities to those of *M. tuberculosis*. It was assumed that they were composed of osmiophilic matter, differing from the nuclear substance. Small, electron-dense particles and large bodies were shown in the cells of *M. lepraemurium* by Whitehouse, *et al.* ([15]). They suggested that the large bodies were most probably deposits of a storage material such as lipid. These large bodies seem to correspond to the inclusions presented in this paper.

One or two sections in serial ultrathin sections (as demonstrated in Fig. 5) sometimes, but not at all frequently, showed fragmentary features of membranous structures near the inclusions. Whitehouse, *et al.* ([15]) stated that the edges of the inclusions of *M. lepraemurium* did not appear well defined. The presence of membranous structures associated with the inclusions is uncertain. The physiological and biochemical natures of these inclusions are yet to be determined. It was supposed by Imaeda and Convit ([3]) that they comprised a polyphosphate, although this had not yet been proved.

There were also some electron-dense, small particles or granules near the cytoplasmic membrane and the inclusions. The inclusions could easily be distinguished from these particles or granules on the basis of their electron density and size. There seemed to be little difference between *M. leprae* and *M. lepraemurium* in the fine structures of the inclusions and electron-dense, small particles or granules at the level of electron microscopic observations by means of serial ultrathin sectioning. However, the cytologic appearance of some of the inclusions observed in *M. lepraemurium* was very diffuse (see Figs. 5 and 6), and these were not seen in *M. leprae* (Figs. 1, 2, and 3). These diffuse ones may be a special feature of murine leprosy bacilli.

Comparative cyto-morphological observations made *in vivo* and *in vitro* will reveal the true biological properties of these inclusions and the electron-dense, small particles or granules in human and murine leprosy bacilli. At this time, it is premature to attempt to reach conclusions based on the results obtained from the electron microscopic observations only.

SUMMARY

The intracytoplasmic inclusions of *Mycobacterium leprae* in human lepromata and *M. lepraemurium* in murine lepromata were studied in ultrathin serial sections at the electron microscopic level. The inclusions were mostly homogeneous and spherical, and did not exist uniformly throughout the bacillary cells. They did not appear to be delimited by membranous structures and apparently had no internal structure. There

seemed to be fundamentally little difference between *M. leprae* and *M. lepraemurium* in the fine structure of these inclusions. However, the large diffuse inclusions observed in the cells of *M. lepraemurium* may be a special feature of murine bacilli.

RESUMEN

Se estudiaron las inclusiones intracitoplásmicas del *Mycobacterium leprae* en lepromas humanos y del *M. lepraemurium* en lepromas murinos por microscopía electrónica en secciones ultradelgadas. Las inclusiones fueron en su mayoría homogéneas y esféricas, no se encontraron uniformemente distribuídas en las células bacilares y no parecieron estar delimitadas por estructuras membranosas ni tener estructura interna. No hubieron diferencias fundamentales en la estructura fina de estas inclusiones entre *M. leprae* y *M. lepraemurium*, aunque las grandes inclusiones difusas observadas en las células del *M. lepraemurium* pueden constituír una característica particular del bacilo murino.

RÉSUMÉ

On a étudié les inclusions intracytoplasmiques de *Mycobacterium leprae* dans les lésions lépromateuses de l'homme, et de *M. lepraemurium* dans des lésions lépromateuses chez le rat, par l'observation au microscope électronique de coupes séquentielles ultra-minces. Pour la plupart, les inclusions étaient homogènes et spériques et n'étaient pas réparties uniformément à travers les cellules bacillaires. Elles n'apparaissaient pas limitées par des structures membraneuses et n'avaient apparemment pas de structure interne. Il a semblé qu'il y avait peu de différences fondamentales entre *M. leprae* et *M. lepraemurium* en ce qui concerne la structure fine de ces inclusions. Néanmoins, les inclusions diffuses de grande dimension observées dans les cellules de *M. lepraemurium* pourraient être une caractéristique spéciale des bacilles murins.

REFERENCES

1. Dobos, R. J. and Hirsch, J. G. *Bacterial and Mycotic Infections of Man.* Philadelphia, Pennsylvania: J. B. Lippincott Company, 1965. Japan: Press of Igaku Shoin, Ltd., Asian ed., 1st printing, 1966.
2. Edwards, R. P. Electron microscope illustrations of division in *Mycobacterium leprae.* J. Med. Microbiol. **3** (1970) 493–499.
3. Imaeda, T. and Convit, J. Electron microscope study of *Mycobacterium leprae* and its environment in a vesicular leprous lesion. J. Bacteriol. **83** (1962) 43–52.
4. Kanai, K. Tetrazolium reducing granules in tubercle bacilli. Kekkaku **36** (1961) 720–726.
5. Kellenberger, E., Ryter, A. and Sechaud, J. Electron microscope study of DNA-containing plasma. II. Vegetative and phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. **4** (1958) 671–678.
6. Kim, K. S. and Barksdale, L. Crystalline inclusions of bacterium 22M. J. Bacteriol. **98** (1969) 1390–1394.
7. Koike, M. and Takeya, K. Fine structures of intracytoplasmic organelles of mycobacteria. J. Biophys. Biochem. Cytol. **9** (1961) 597–608.
8. Kushida, H. On n-butyl methacrylate ethyl methacrylate embedding. Electron Microscopy Japan **5** (1957) 128.
9. Lembke, A. and Ruska, H. Vergleichende mikroskopische und über mikroskopische Beobachtungen an den Erregern der Tuberkulose. Klin. Wochenschr. **19** (1940) 217–220.
10. Mudd, S., Takeya, K. and Henderson, H. J. Electron-scattering granules and reducing sites in mycobacteria. J. Bacteriol. **72** (1956) 767–783.
11. Smith, D. T. and Conant, N. E. *Zinsser Microbiology.* New York, New York: Appleton Century Crofts, Inc., 1960.
12. Toda, T., Koike, M., Hiraki, N. and Takeya, K. The intracellular structure of a mycobacterium. J. Bacteriol. **73** (1957) 442–443.
13. Weineb, S. Ultraviolet polymerization of monomeric methacrylates for electron microscopy. Science **121** (1955) 774–775.
14. Wessel, E. Über mikroskopishe Beobachtungen an Tuberkel Bazillen vom *Typus humanus.* Z. Tuberk. **88** (1942) 22–36.
15. Whitehouse, R. L. S., Wong, P. C. and Jackson, F. L. Ultrastructure of *Mycobacterium lepraemurium.* Int. J. Lepr. **39** (1971) 151–163.