THE PROBLEM OF PRESERVING INTERNAL STRUCTURES IN PATHOGENIC MYCOBACTERIA BY CONVENTIONAL METHODS OF FIXATION

John H. Hanks, Ph.D.

Leonard Wood Memorial Laboratory
Department of Bacteriology and Immunology
Harvard Medical School, Boston, Mass.[1]

Since the earliest days of microbiology, evidence has accumulated that the pathogenic mycobacteria differ from saprophytic mycobacteria and other types of microbes because of an impermeability which confers resistance to drying and to acids, alkalies, cationic wetting agents, dyes, and other chemical agents. These properties are due to the inert capsules and matrices which they possess. In any suspension, these structures are least permeable on the cells which are in optimal physiological state ([5]). That such structures may prevent penetration by the conventional "fixatives," and that fixatives may increase the impermeability, seems not to have been recognized. The importance of this oversight can be illustrated in several ways.

Simple drying tends to preserve tissue cells, bacteria or saprophytic mycobacteria in films on glass slides. Such preparations stain satisfactorily and reveal the original numbers of cells or bacteria after many months of storage at room temperature. When comparable films containing *Mycobacterium leprae* from scraped skin incisions were "fixed" in several ways, then stored for one month at room temperature prior to carbol-fuchsin staining, the results were as shown in Table 1.

It is seen that various proportions of the bacilli demonstrable before storage disappeared during the month of storage under the conditions specified. Since many "sausage skin" remnants were visible in surface-coated preparations ([5]), it is assumed that autolysis occurred. The proneness of *M. leprae* to disappear after presumed fixation has been reported previously ([4]) and noted by other workers.

It is known that formalin fumes or solutions impart to tissue cells an excessive basophilism; also that formalin excels other fixatives in promoting retention of ribose nucleic acids in naked bacterial spheroplasts ([2]). When dry films containing tissue cells, dried *M. phlei*, or serum proteins are placed in a sealed container in which 1 per cent of the volume is occupied by formalin (40% formaldehyde solution), excessive basophilism is imparted within less than 3 minutes. Such ex-

[1] New address: Johns Hopkins—Leonard Wood Memorial Leprosy Research Laboratory, Department of Pathobiology, The Johns Hopkins School of Hygiene, Baltimore 5, Maryland.

TABLE 1.—*Autolysis of* M. leprae *after "fixation."*

Heat fixation [a]	Post-fixation	Condition of storage (1 month)	Per cent of original bacilli demonstrated (averages)
98°C, 2 min.	(None) 95% alcohol, 5 min. 10% formol-alcohol,[b] 30 min.	Relative humidity ±20%[c]	10 34 (29) 44
98°C, 2 min.	(None) 95% alcohol, 5 min. 10% formol-alcohol,[b] 30 min.	Desiccated over Drierite	52 56 (56) 59

[a] On a glass plate over a boiling waterbath; possibly equivalent to flame fixation.
[b] Formaldehyde solution, 1 part, plus 95 per cent alcohol, 9 parts.
[c] Heated room, northerly climate, March.

posures, however, suffice only to make *M. leprae murium* much less dye-permeable than it was originally. Exposure to the formalin fumes must be continued for 6 hours at 37°C before basophilism is accentuated.

Optimal conditions for the fixation of internal structures in tissue cells and bacteria prior to the preparation of ultra-thin sections for electron microscopy have been investigated by many workers. Conventional periods of fixation in buffered 2 per cent osmium tetroxide have permitted the demonstration of internal structures in the more permeable mycobacteria. There has been, however, a general failure to demonstrate in pathogenic mycobacteria the internal structures of functional cells. The first adequate representation of such structures was in a paper by Yoshida *et al.* ([8]) who demonstrated the necessity of prolonged or repeated fixation. Shinohara *et al.* ([7]) found it necessary to fix *M. avium* for 2 days, and H-37Rv for 5 days. In our studies with Chapman ([1]) on the fixation of internal structures in *M. leprae murium*, no satisfactory images were obtained until fixation in 2 per cent osmium tetroxide had proceeded for 6 days at pH 8.5. This interval, of course, exceeded greatly that which was suitable for tissue cells in the preparations.

In experiments on the permeability of pathogenic species to dyes ([5]) it repeatedly has been observed that immersion for 5-10 minutes in 10 per cent formalin or 95 per cent alcohol decreases significantly the normal rates of dye permeation. Similar observations with other fixing solutions may show that the earliest effect of many fixatives is to impede their own penetration.

DISCUSSION

In view of the foregoing observations, two questions must be raised: (1) In the long history of cytologic and histologic study of materials

containing pathogenic mycobacteria, how well have the chemical fixatives accomplished the limited objective of retaining the gross structures and original numbers of bacilli? (2) How are such mycobacteria to be fixed for study of both gross and fine structure without over-fixation of accompanying cells and tissue structures?

On the problem of fixing structures within encapsulated (pathogenic) mycobacteria, suggestions may be made. In the first place, prolonged fixation does not provide an ideal answer, since alterations of internal structure may occur before the fixative can act. It seems, on the contrary, that the best principle would be to expose the organisms to the fixative while subjecting these systems to conditions which relieve impermeability. Impermeability decreases very slowly during incubation at 37°C ([5]), more rapidly in dilute phenol ([6]), and very rapidly during heating to 98°C or shaking with 5-10 per cent chloroform in aqueous suspension ([5]). The effect of wetting agents other than Tween 80 (not effective), combined with dyes or fixatives, has not been examined, although there is a suggestion that useful effects should be obtained ([3]).

Since the time required to modify permeability differs greatly among the cells in a population ([5]), it may be emphasized that a dye or fixative should be present to stabilize structures as rapidly as they may be exposed to external factors. The use of dyes to assay the integrity of internal (presumed nuclear) granules has demonstrated that treatment with heat or chloroform in the presence of water destroys capsules and distorts internal structures. If, however, the organisms are heated to 98°C for 2-3 minutes or at 60°C for 30-40 minutes in the presence of 0.4 per cent safranin 0 or 0.04-0.4 per cent crystal violet, the internal granules are nicely preserved. At 50°C for 30 minutes, only some 50 per cent of the cells of *M. leprae murium* are made sufficiently permeable to acquire dye.

While fixation of internal structures in pathogenic mycobacteria might be faciliated by judicious use of phenol, chloroform, wetting agents or heat in the presence of chemical fixatives, two problems remain: the modification or loss of capsules, and the question of simultaneous preservation of structure in tissue cell components.

SUMMARY

This note presents evidence that, because of capsules and matrices on pathogenic mycobacteria, the first effect of several popular "fixatives" is to impede their own penetration. A suggestion is made that rapid and more satisfactory preservation of internal structures might be achieved by exposures to fixatives in the presence of heat, phenol, or chloroform as agents which modify the impermeability of capsular structures.

RESUMEN

Ofrécense pruebas de que, debido a las cápsulas y matrices de las micobacterias patógenas, el primer efecto de varios "fijadores" en boga consiste en impedir su propia penetración. Se presenta la indicación de que cabría obtener una conservación rápida y más satisfactoria de los tejidos internos con la exposición a fijadores en presencia de calor, fenol o cloroformo como agentes que modifican la impermeabilidad de las estructuras capsulares.

RESUMÉ

Cette communication fournit des arguments pour démontrer que, par suite de l'existence de capsules et de matrices entourant les mycobactéries pathogènes, le premier effet de plusieurs "fixateurs" communément utilisés est d'empêcher leur propre pénétration. L'auteur suggère qu'une préservation rapide et plus satisfaisante des structures internes pourrait être réalisée par l'exposition aux fixateurs en présence de la chaleur, du phenol, ou de chloroforme, ces agents visant à modifier l'imperméabilité des structures capsulaires.

REFERENCES

1. CHAPMAN, G. B., HANKS, J. H. and WALLACE, J. H. An electron microscope study of the disposition and fine structure of *Mycobacterium leprae murium* in mouse spleen. J. Bact. **77** (1959) 205-211.
2. FITZ-JAMES, P. C. Studies on the morphology and nucleic acid content of protoplasts of *Bacillus megaterium*. J. Bact. **75** (1958) 369-382.
3. GRAY, J. B. Personal communication, 1958.
4. HANKS, J. H. The fate of the bacilli in incubated lepromatous tissues and the question of microscopic growth. Internat. J. Leprosy **13** (1945) 9-24.
5. HANKS, J. H. Significance of capsular components of *Mycobacterium leprae* and other mycobacteria. Internat. J. Leprosy **29** (1961) 74-83.
6. HANKS, J. H. Demonstration of capsules on *M. leprae* during carbolfuchsin staining; mechanism of the Ziehl-Neelsen stain. Internat. J. Leprosy **29** (1961) 179-182.
7. SHINOHARA, C., FUKUSHI, K., SUZUKI, J. and SATO, K. Mitochondrial structure of *Mycobacterium tuberculosis* relating to its function. J. Electronmicroscopy **6** (1958) 47-52.
8. YOSHIDA, N., FUKUI, K., TAMAKI, T., TANI, A., HASHIMOTO, Y., HARA, Y., TSUYOSHI, M., and KAWANO, A. Studies on the bacterial mitochondria and cristae mitochondriales. II. Electron microscopical studies of ultra-thin sections of Mycobacterium tuberculosis var. hominis. Shikoku Acta Medica, **11** (1957) 190-194.