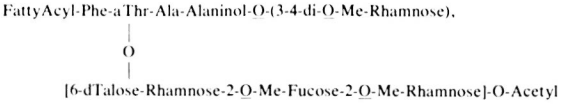
INTERNATIONAL JOURNAL OF LEPROSY Volume 48, Number 4
Printed in the U.S.A.

Evidence for Species-Specific Lipid Antigens in *Mycobacterium leprae*[1]

Patrick J. Brennan and William W. Barrow[2]

Electron microscopic examination of tissue infected with *Mycobacterium leprae* has established the presence of an electron-transparent zone surrounding the bacilli *in situ* ([10, 22, 23]). A similar zone was also observed in mouse tissue infected with *M. lepraemurium* ([4, 5, 8, 9, 14]). It has been suggested that these zones represent a mechanically protective "capsule" which may serve to insulate these pathogens within phagolysosomes from lysosomal enzymes. In fact, a "capsule" was isolated by Draper and Rees ([9]) from *in vivo* cultivated *M. lepraemurium*, which apparently is the basis of the electron-transparent zone. Electron microscopy of the negatively stained isolated "capsules" from *M. lepraemurium* showed a most characteristic pattern of tape-like fibers and sheets, and preliminary chemical analyses on the capsular substance revealed a few select amino acids (phenylalanine, threonine, alanine), fatty acids, and 6-deoxysugars, suggesting that the "capsule" contained "C-mycosidic" peptidoglycolipids ([9]). In recent work we isolated from an *M. intracellulare* (serovar 20 or the *M. avium/M. intracellulare/M. scrofulaceum* sero complex) material which was morphologically identical to the "capsules" from *M. lepraemurium* ([1]). The tape-like fibrils were almost entirely composed of polar "C-mycosidic" peptidoglycolipids (PGLs) with the structure:



Indeed, from other work of ours we conclude that all of the 30 odd serovars in the *Mycobacterium avium/M. intracellulare/M. scrofulaceum* (MAIS) complex and other "atypical" smooth colony mycobacteria are endowed with a capsule of exquisitely type specific polar PGLs (Brennan, P. J., unpublished observations). Moreover these PGLs are serologically active; they are the only bacterial substances which produce precipitin reactions in agar gels when tested against antisera from rabbits immunized with the homologous serovar. Type-specificity resides largely in the threonine-linked oligosaccharide because the lipopeptide portion is relatively invariant from one organism to another whereas the appended oligosaccharides vary in size and sugar composition.

There is some circumstantial evidence to suggest that *M. leprae in situ* are endowed with a "capsule" composed of lipids. Hanks ([13]), working with *M. leprae* infected tissue, was probably the first to refer to the electron transparent zone as a "bacterial capsule," and Sato and Imi ([20]) recognized that the zone may surround single bacilli or clusters of them. They called it a "network pattern consisting of tape-like fibers" and compared it to the "peripheral halo" described by Malfetti ([16]). From staining properties during electron microscopic examination, Nishiura ([18]) concluded that the zone around *M. leprae* is lipid. In his review Ratledge ([19]) referred to a "large lipoidal zone or capsule surrounding *M. leprae* and *M. lepraemurium*." In a recent

[1] Received for publication on 26 June 1980; accepted for publication on 25 August 1980.

[2] P. J. Brennan, Ph.D., Senior Research Scientist, National Jewish Hospital and Research Center, Denver, Colorado 80206 and Associate Professor, Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523, U.S.A.; W. W. Barrow, Ph.D., Post-doctoral Fellow of the Heiser Fellowship Program for Research on Leprosy, National Jewish Hospital and Research Center, Denver, Colorado 80206, U.S.A. Dr. Barrow's present address is Department of Microbiology and Immunology, Texas College of Osteopathic Medicine, Fort Worth, Texas 76107, U.S.A.

comparative ultrastructural examination Nguyen, *et al.* [17] concluded that the cell envelope of *M. leprae* and *M. lepraemurium* differ particularly with respect to the amount and complexity of "the superficial peptidoglycolipid and mycosidic integument," which was poorly developed in *M. leprae*.

Our current studies amount to a quest for species-specific lipid antigens in *M. leprae*. If such antigens exist, we hope that they can be utilized in a useful manner such as in the development of diagnostic skin tests and serological tests for leprosy.

MATERIALS AND METHODS

Isolation of *M. leprae* from infected armadillo liver. Livers from infected armadillos were obtained from Dr. W. F. Kirchheimer, U.S. Public Health Service Hospital, Carville, Louisiana through Dr. D. D. Gwinn, Leprosy Program Officer of the National Institutes of Allergy and Infectious Diseases. The Number of acid-fast bacilli per g of liver ranged from 1.5×10^7 to 1.0×10^{10}. For the isolation of *M. leprae* we have relied on the "gentle method" developed by Dr. Philip Draper [7].

Extraction of lipids from *M. leprae*. Two procedures were used for removal of lipids from purified *M. leprae*. Lyophylized cells (100 mg) were extracted twice with 2–3 ml of acetone at room temperature for 18 hr and centrifuged. The supernatant contained the acetone-soluble lipids. Acetone extracts of *M. leprae* were also given to us by Dr. T. M. Buchanan, U.S. Public Health Service Hospital, Seattle, Washington.

Lyophylized *M. leprae* were also extracted with chloroform-methanol (2:1) at 50°C for 18 hr [2]. The extract was centrifuged to yield a supernatant containing total soluble lipids.

Chromatographic and chemical procedures. Lipid was applied to columns (1×15 cm) of silicic acid-Celite (2:1), prepared in $CHCl_3$, which were then developed with increasing concentrations of CH_3OH in $CHCl_3$. Eluates were evaporated to dryness with N_2 and used for serology and acid or alkaline degradation.

Lipids were hydrolyzed in a small volume (100–200 μl) of a solution containing concentrated HCl (36.5–38%; 10 ml), water (55 ml), and glacial CH_3COOH (35 ml) at 94°C steam for 7 hr. The hydrolysate was extracted with hexane to remove fatty acids, and the aqueous phase was neutralized and dried with the aid of a stream of N_2 [2]. The residue was dissolved in water and passed through a column of mixed bed resin (MB-3) before chromatography of sugars. Sugars as their alditol acetates were subjected to gas liquid chromatography (GLC) as described previously [2]; further details are given in the legend to Fig. 2.

Lipids were also treated with alkali as described before [3]. In essence, 0.2 N NaOH in CH_3OH was added to an equal volume of lipid in $CHCl_3$-CH_3OH (2:1). After 20 min at 37°C, the mixture was neutralized with CH_3COOH, dried, and partitioned between $CHCl_3$-CH_3OH (2:1) and water. The $CHCl_3$ phase contained the alkali-stable lipids.

Serology. The micro-gel diffusion technique described by Crowle [6], using gels prepared from 1 percent agarose in 0.04 M Veronal buffer pH 7.4, was used. Lipid preparations were evaporated to dryness and suspended by sonicating for 4 min in phosphate buffered saline (PBS) before applying to wells in the agar blocks. Occasionally, in order to facilitate suspension, lipids were treated with 0.5 percent Tween in PBS before applying to gels previously immersed for 24 hr in the detergent.

Serum from normal and *M. leprae*-infected armadillos were obtained from the source which provided infected livers. Sera from the two lepromatous patients originated in Brazil from biopsy proven cases and were given to us by Dr. M. H. Kaplan, North Shore University Hospital, New York. Sera from a patient with tuberculosis and another patient with *M. avium* infection were also generously supplied by Dr. Kaplan. The sera from all patients were known to have either LEP antigen [15] or tuberculopolysaccharide (information provided by Dr. Kaplan).

RESULTS AND DISCUSSION

Isolation of *M. leprae*. The procedure which we used for purification of *M. leprae* is almost identical to the "gentle method" developed by Dr. Philip Draper [7]. Points of note with regard to our application of

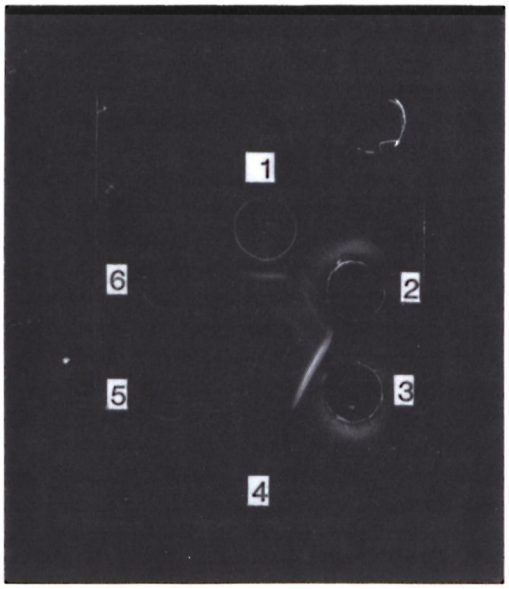


FIG. 1. Agar gel immunodiffusion (detergent not included) of the lipid fraction from *M. leprae* which had been eluted from silicic acid with 5 percent CH_3OH. To the center well was added 96 μg of the lipid suspended in 20 μl PBS. Each of the peripheral wells contained 20 μl of serum. 1. Serum from *M. leprae* infected armadillo. 2. Serum from lepromatous patient 3. 3. Serum from lepromatous patient 16. 4. Serum from normal armadillo. 5. Serum from *M. avium* patient. 6. Serum from a tuberculosis patient.

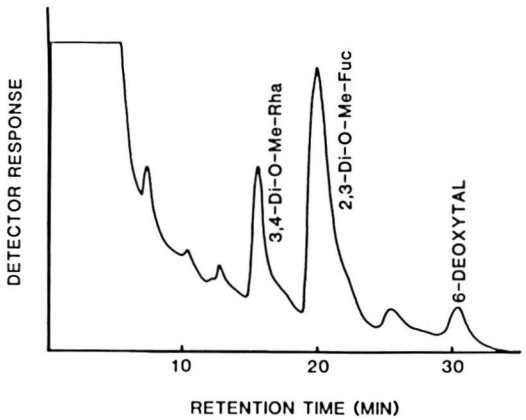


FIG. 2. GLC of the sugars present in the 5 percent CH_3OH lipid fraction. About 0.5 mg of the lipid was hydrolyzed, reduced with $NaBH_4$, and acetylated. Operating conditions were 3 percent SP-2340 on 100/120 Supelcoport, 167°, 60 ml N_2/min.

Draper's procedure are that the optional step involving collagenase digestion was never included (the only enzymatic digestion step involved DNAase), a gradient with 30 percent Percoll was used, and 40 g of infected tissue was processed at any one time. An important consideration is that the load of bacilli in infected tissue should be at least 10^9/g.

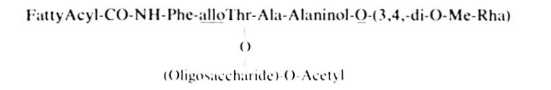
Between December 1979 and March 1980 almost 1 kg of infected armadillo liver was processed, and over 2 g of purified *M. leprae* were recovered. Yield of bacilli was less than that reported by Draper [7]. However, the bacilli looked excellent; smears of the final preparation, when stained with the soluble blue preparation of Wheeler and Draper [21], appeared to be devoid of nonbacterial contaminants.

Search for *M. leprae* specific lipid antigens. The approach used in the search for antigens specific to *M. leprae* emulated that developed for atypical mycobacteria [2]. Lipid extracts obtained from *M. leprae* with either acetone or $CHCl_3$-CH_3OH were sonicated and tested for serological activity by gel diffusion against antisera from patients with lepromatous leprosy or other mycobacterial infections. Both types of lipid extracts gave distinct lines of precipitation with the antisera from lepromatous patients but did not react with the sera from patients with tuberculosis or *M. avium*.

These lipid extracts were next applied to columns of silicic acid-Celite which were eluted with $CHCl_3$, followed by steps of 1 percent through 5 percent CH_3OH in $CHCl_3$, and lastly by 10 percent CH_3OH in $CHCl_3$. Each eluate was dried, sonicated in PBS, and again tested for serological activity. Usually with such chromatograms activity was located only in the 5 percent CH_3OH fraction. As shown in Fig. 1, this activity was markedly specific in that it applied only to antiserum from lepromatous patients and *M. leprae* infected armadillo and did not extend to serum from normal armadillos and from patients with tuberculosis or *M. avium* infection. We wish to emphasize that these are preliminary experiments. The activity of such lipid preparations has yet to be tested against antisera from a greater number and variety of patients with leprosy and other mycobacterial infections.

Clues on the chemical nature of the sero-

logically active lipids from *M. leprae*. Recent evidence from this laboratory ([2]); (Brennan, P. J., Mayer, H., Aspinall, G. O. and Nam Shin, J. E., submitted for publication) has shown that the type specific (so called "typing") antigens from a wide range of mycobacteria are polar C-mycosidic peptidoglycolipids (PGL) conforming to the generic structure:



A characteristic feature of these lipid antigens is that they are alkali-stable. In other words, when treated with 0.2 N NaOH they remain as lipids because, despite the loss of the acetyl substituents, the amide linked fatty acyl and the rest of the molecule remain intact. The dominant sugar species in the oligosaccharide segment are 6-deoxyhexoses because 6-deoxytalose is invariably the linkage terminus, rhamnose is invariably penultimate, and other variable 6-deoxyhexoses may occupy the outermost segment of the oligosaccharide.

With the possibility in mind that the specific serologically active lipids from *M. leprae* may be related to the C-mycosidic PGLs, we first treated the lipid fraction which had been eluted with 5 percent CH_3OH in $CHCl_3$ with 0.2 N NaOH and examined the ensuing alkali stable lipid for serological activity. Activity remained, indicating that the responsible lipid was indeed alkali stable. We then sought 6-deoxyhexoses in the 5 percent CH_3OH fraction. Portions of the lipid were hydrolyzed with a solution containing HCl and CH_3COOH, and the resulting sugars were converted to their alditol acetates and examined by gas liquid chromatography (GLC) (Fig. 2). The retention times of the peaks were highly suggestive of 6-deoxyhexoses, and co-chromatography with the alditol acetates arising from the C-mycosidic PGL from *M. intracellulare* serovar 9 (Brennan, P. J., Mayer, H., Aspinall, G. O. and Nam Shin, J. E., submitted for publication) suggested that three of the peaks were 3,4-di-O-methyl-rhamnose, 2-3-di-O-methyl-fucose, and 6-deoxytalose.

Of course, we are not yet in a position to state that the 6-deoxyhexose-containing lipids are the basis of the specific serological activity associated with *M. leprae* lipids; a pure active lipid has yet to be isolated and thoroughly characterized. At this time we can merely state that there is indirect evidence implicating 6-deoxyhexose-containing lipids with this serological activity. Neither are we prepared to state that the 6-deoxyhexose-containing lipids are C-mycosides. We know from experience that removal of C-mycosidic PGLs from silicic acid columns requires more than 5 percent CH_3OH. Therefore mycosides of the A, B or G variety—which also contain 6-deoxyhexoses and are alkali-stable and less polar than the C-mycosides—are possible candidates for the role of specific antigens on the surface of *M. leprae* (for a recent review of mycoside structure see Goren and Brennan ([12])). It is interesting to recall that in 1973 M. Gastambide-Odier ([11]) stated, perhaps with some vision, "Les résidus glycosidiques spécifiques des mycosides A, B et G pourraient être les groupements determinants d'haptènes . . . ."

Currently we are also looking for this lipid "antigen" in liver fractions left after *M. leprae* have been removed. The logic here is that if cold acetone will solubilize the antigen, then much of it may have been lost during the fractionation steps involved in the isolation of *M. leprae*. Moreover, Sato and Imi (20) noted that the surface structure of *M. leprae* is most fragile and liable to fragmentation. We hope that chemical characterization of the antigen will be followed by the preparation of a semi-synthetic antigen which may then be used for the development of diagnostic skin tests and serological tests for leprosy and in studies of cell mediated immunity and correlates of resistance.

SUMMARY

A partially purified lipid fraction from *Mycobacterium leprae* yielded distinct lines of precipitation with antisera from two lepromatus patients and from an infected armadillo. There was no reaction to the sera from patients with tuberculosis or a *M. avium* infection or to the serum from a normal armadillo. The activity in the lipid fraction was unaffected by mild alkali,

and upon hydrolysis the fraction yielded 6-deoxyhexoses. This information suggests that the lipid antigens of *M. leprae* may be species specific and related to the A, B, C or G mycosides.

RESUMEN

Una fracción lipídica parcialmente purificada del *Mycobacterium leprae* produjo claras lineas de precipitación con los antisueros de dos pacientes lepromatosos y con el suero de un armadillo infectado. No hubo reacción con los sueros de pacientes con tuberculosis, con el suero de un paciente infectado por *M. avium*, ni con el suero de un armadillo normal. La actividad de la fracción lipídica no se afectó por el tratamiento moderado con álcali. La hidrólisis del lípido liberó 6-desoxihexosas. Esta información sugiere que los antígenos lipídicos del *M. leprae*, pueden ser específicos de la especie y estar relacionados con los micósidos A, B, C o G.

RÉSUMÉ

Une fraction lipidique partiellement purifiée de *Mycobacterium leprae* a permis de mettre en évidence des bandes distinctes de précipitation par des antisera provenant de deux malades lépromateux et d'un armadillo infectieux. On n'a pas observé de réaction à l'égard du sérum provenant de patients atteints soit de tuberculose, soit d'une infection par *M. avium*, pas plus qu'on a pu observer de réactions à l'égard du serum obtenu chez un armadillo normal. L'activité de la fraction lipidique n'était pas affectée par une légère alcalinisation du milieu. A la suite d'hydrolyse, la fraction lipidique a livré des 6-dioxyhexoses. Ces résultats suggèrent que les antigènes lipidiques de *M. leprae* pourraient être spécifiques en ce qui concerne l'espèce, et en relation avec les mycosides A, B, C ou G.

Acknowledgements. This work was funded by a Contract (AI-92625) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. William Barrow, Ph.D. is a recipient of a Postdoctoral Fellowship from the Heiser Fellowship Program for Research on Leprosy. We wish to thank Ms. Beth Ullom for skillful technical assistance and also thank Dr. Darrel D. Gwinn of the National Institutes of Health for encouragement and active help in the early stages of this program.

REFERENCES

1. Barrow, W. W., Ullom, B. P. and Brennan, P. J. The peptidoglycolipid nature of the superficial cell wall sheath of smooth colony forming mycobacteria. J. Bacteriol. (in press).
2. Brennan, P. J. and Goren, M. B. Structural studies on the type-specific antigens and lipids of the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* serocomplex. J. Biol. Chem. **254** (1979) 4205–4211.
3. Brennan, P. J., Souhrada, M., Ullom, B., McClatchy, J. K. and Goren, M. B. Identification of atypical mycobacteria by thin-layer chromatography of their surface antigens. J. Clin. Microbiol. **8** (1978) 374–379.
4. Brown, C. A. and Draper, P. An electron-microscope study of rat fibroblasts infected with *Mycobacterium lepraemurium*. J. Pathol. **102** (1970) 21–26.
5. Chapman, G. B., Hanks, J. H. and Wallace, J. H. An electron microscope study of the disposition and fine structure of *Mycobacterium lepraemurium* in mouse spleen. J. Bacteriol. **77** (1959) 205–211.
6. Crowle, A. J. *Immunodiffusion*. 2nd ed. New York: Academic Press, 1973, pp. 247–303.
7. Draper, P. 1979. Annex 1, Protocol 1/79, Report of the Enlarged Supervisory Council Meeting for Research on the Immunology of Leprosy, World Health Organization, Geneva, 7–8 February.
8. Draper, P. and Rees, R. J. W. Electron-transparent zone of mycobacteria may be a defense mechanism. Nature **228** (1970) 860–861.
9. Draper, P. and Rees, R. J. W. The nature of the electron-transparent zone that surrounds *Mycobacterium lepraemurium* inside host cells. J. Gen. Microbiol. **77** (1973) 79–87.
10. Edwards, R. P. Electron-microscope illustrations of division in *Mycobacterium leprae*. J. Med. Microbiol. **3** (1970) 493–499.
11. Gastambide-Odier, M. Variantes de mycosides charactérisées par des résidues glycosideques substitués par des chaines acyles. Eur. J. Biochem. **33** (1973) 81–86.
12. Goren, M. B. and Brennan, P. J. Mycobacterial lipids: chemistry and biological activities. In: *Tuberculosis*. Youmans, G. P., ed. Philadelphia: W. B. Saunders Co., 1979, pp. 63–193.
13. Hanks, J. H. The origin of the capsules on *Mycobacterium leprae* and other tissue-grown mycobacteria. Int. J. Lepr. **29** (1961) 172–174.
14. Hasegawa, T. Further electron microscopic observations of *Mycobacterium lepraemurium*. Dermatologica **143** (1971) 363–367.
15. Kaplan, M. H. and Chase, M. W. The dynamics of antibody responses to 9 defined mycobacterial antigens throughout the course of treatment of 52 tuberculosis patients. Proc. 14th Joint conference on tuberculosis, U.S.–Japan Cooperative Medical Science Program, Denver, Co. 1979, pp. 498–527.
16. Malfetti, M. G. Study of the morphology of *Mycobacterium leprae* under the electron microscope. Int. J. Lepr. **20** (1952) 94–104.
17. Nguyen, H-T., Trach, D. D., Van Man, N., Ngoan, T. H., Dunia, I., Ludosky-Diawara, M. A. and Benedetti, E. L. Comparative ultrastructure of *Mycobacterium leprae* and *Mycobac-*

terium lepraemurium cell envelopes. J. Bacteriol. **138** (1979) 552–558.

18. Nishiura, M. The electron microscopic basis of the pathology of leprosy. Int. J. Lepr. **28** (1960) 357–400.
19. Ratledge, C. The physiology of mycobacteria. Adv. Microbiol. Physiol. **13** (1976) 116–245.
20. Sato, S. and Imi, M. The surface structure of *Mycobacterium leprae*. Int. J. Lepr. **36** (1968) 303–308.
21. Wheeler, P. R. and Draper, P. Soluble blue as a counterstain in the Ziehl-Neelsen procedure. Int. J. Lepr. **48** (1980) 15–17.
22. Yamamoto, T., Nishiura, M., Harada, N. and Imaeda, T. Electron microscopy of *Mycobacterium lepraemurium* in ultrathin sections of murine leprosy lesions. Int. J. Lepr. **26** (1958) 111–114.
23. Yoshizumi, M. D. and Ashbury, A. K. Intra-axonal bacilli in lepromatous leprosy. Acta. Neuropathol. **27** (1974) 1–10.