Lipids in Leprosy

I. Histochemistry of Lipids in Murine Leprosy[1, 2]

Isamu Sakurai and Olaf K. Skinsnes[3]

Murine leprosy is analogous to human lepromatous leprosy by similarities in host cell reactions and some characteristics of causative agents. As in the lesions of lepromatous leprosy so also in the lesions of murine leprosy there are innumerable undigested bacilli and large amounts of lipids in macrophages.

The present studies were initiated as a part of a series of analysis of the chemical compositions of intracellular lipids and their genesis in the reticuloendothelial cells of murine leprosy as compared with those in human leprosy ([19]).

MATERIALS AND METHODS

Experimental animals. Twenty-four inbred female mice of the C_3H strain were infected with *Mycobacterium lepraemurium* and four were used as controls. The mice were 12 to 14 weeks old at the time of inoculation.

Bacillary inoculum. The Hawaiian strain of *Mycobacterium lepraemurium* was obtained from lepromas of a maintained stock infected mice of same strain. The lepromas were minced with sterile scissors and homogenized in physiologic saline in a sterile Potter-Elvehjem tissue grinder. The homogenates were centrifuged at 1,500 rpm for 20 minutes to remove tissue fragments. The supernatant crude suspension was used for inoculation.

The experimental animals were inoculated intraperitoneally with 0.5 ml. of the crude suspension of *M. lepraemurium*. Mice were divided into six groups of four inoculated mice each and sacrificed at 2, 4, 6, 8, 12 and 16 weeks after inoculation.

Four control animals were injected intraperitoneally with 0.5 ml. of physiologic saline, and sacrificed at 16 weeks.

Peritoneal spread. The omentums and mesenteries were obtained from the sacrificed animals and were spread on glass microscope slides, partially dried for several minutes and fixed with 10 per cent neutralized formalin or formol-calcium solution for phospholipid staining.

Spleen. The spleens were removed and similarly fixed. Blocks of spleen from each animal were embedded in paraffin, and in carbowax (polyethylene glycol, Union Carbide Corporation) ([17]), for lipid stains after fixation was completed.

Routine stains. Methylene blue and Ziehl-Neelsen's stains were employed for the peritoneal spreads.

Hematoxylin and eosin stain, and Ziehl-Neelsen's stains were employed for the formalin-fixed, paraffin-embedded tissues.

Lipid histochemistry. Various methods for the identification of each lipid were applied to sections suitably fixed for each method. Sections were cut on a rotary microtome at 6 to 10 microns in a low humidity, air-conditioned room with temperature at approximately 22°C.

The histochemical procedures employed for lipid identifications were largely according to methods detailed by Pearse ([16]) and Okamoto *et al.* ([15]). They consisted of the following:

I. Lipid in general and neutral fat:
   (a) Sudan III (Daddi's method)
   (b) Fett rot
   (c) Nile blue sulfate

II. Fatty acid:
   (a) Fischler's method

[1] Received for publication 9 April 1970.
[2] This work was supported by Grant AI-08878 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014.
[3] I. Sakurai, M.D., Sc.D., Assistant Researcher, and O. K. Skinsnes, M.D., Ph.D., Professor, Department of Pathology, University of Hawaii School of Medicine, Leahi Hospital, 3675 Kilauea Avenue, Honolulu, Hawaii, 96816. *Dr. Sakurai's present address*: Department of Pathology, Nihon University School of Medicine, Itabashi-ku, Tokyo, Japan.

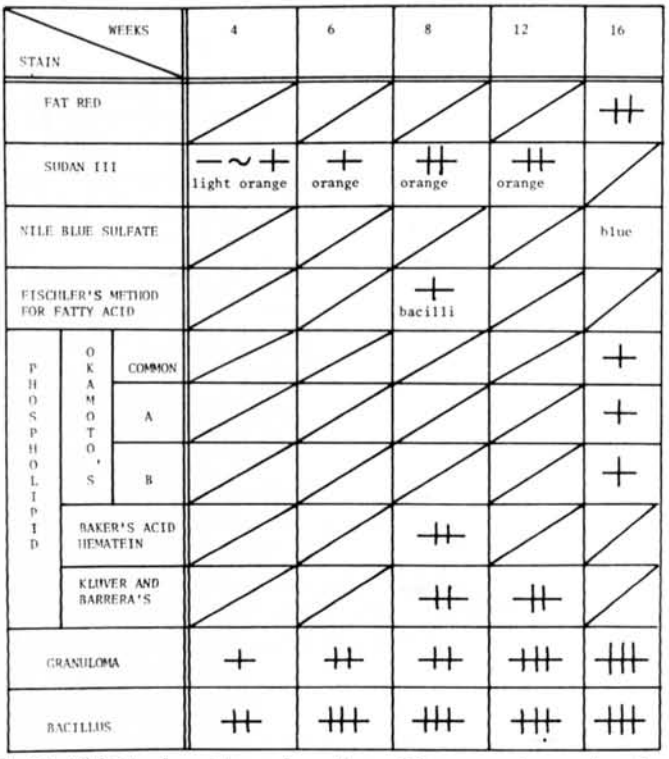

FIG. 1. Lipid histochemistry of peritoneal leproma in murine leprosy.

III. Cholesterol and its ester:
  (a) Okamoto *et al.* sulfuric acid method
  (b) Okamoto *et al.* acetic acid-sulfuric acid method
IV. Glycolipid:
  (a) Okamoto *et al.* modified Molisch reaction
V. Phospholipid:
  (a) Okamoto *et al.* mercury diphenylcarbazone method
  (b) Baker's acid hematein method (formol-calcium fixation)
  (c) Klüver-Barrera's copper phthalocyanin method (formol-calcium fixation)
VI. Firmly bound lipid:
  (a) Sudan black B stain after extraction by chloroform-methanol (2:1) mixture, together with extraction by acidified chloroform-methanol mixture (1% conc. hydrochloric acid in chloroform-methanol, 2:1, mixture) as negative control.

RESULTS

Peritoneal spread (Fig. 1). All histochemical methods could not be applied to tissues of each mouse since in the earlier stages of infection not enough infected peritoneal tissue was available from each animal. In animals sacrificed at four to 16 weeks, however, peritoneal lepromas were increasingly available.

By four weeks after inoculation, small granulomas in the peritoneal specimens began to be formed around small blood vessels. Macrophages in the lepromas contained fairly large numbers of bacilli, and some of the macrophages were stained a light orange by Sudan III, while others were not.

By six weeks, the lepromas were increased in number and size. Most macrophages stained with Sudan III and fett rot. The color was not the bright red shown by fat cells but a yellow-orange. The color reaction shown with Sudan III stain suggests that the lipids in the macrophages are probably composed mainly of lipoid sub-

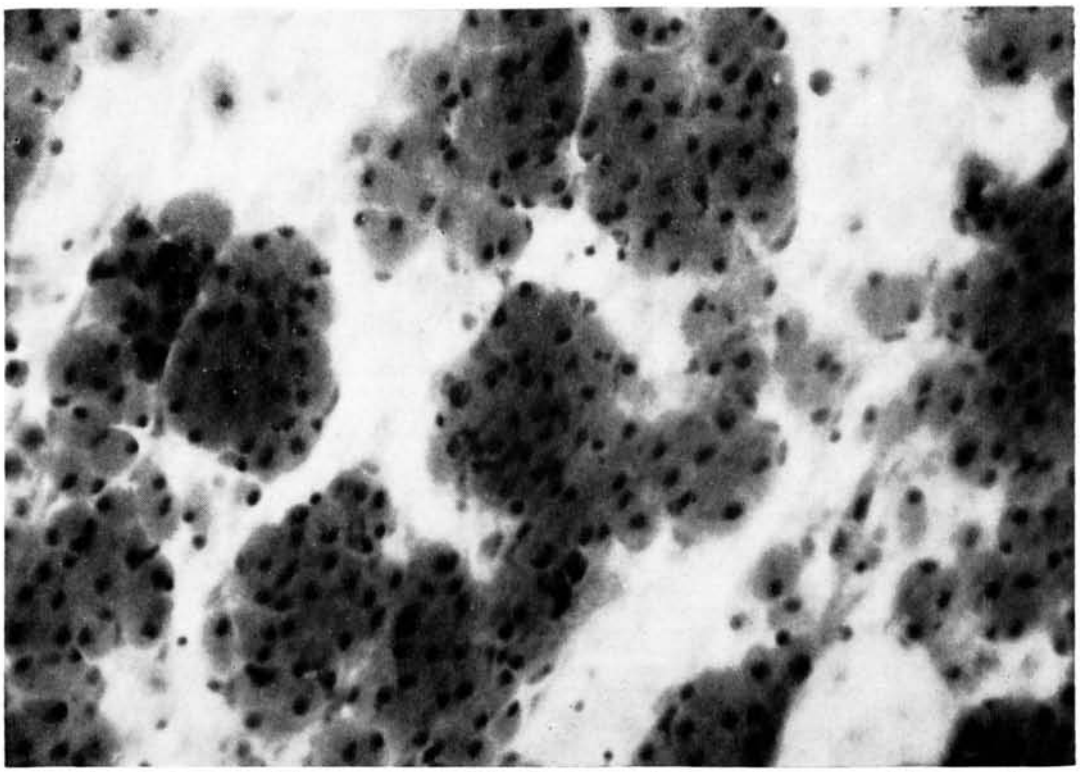

FIG. 2. Peritoneal lepromas stained by Klüver and Barrera's copper phthalocyanin method for phospholipids (12 weeks after inoculation). Original magnification X100.

stance other than neutral fat, which should be stained bright red with Sudan III. The bacilli were more numerous in the lepromas.

By eight weeks the lepromas were progressively increased in size and number, and bacilli were correspondingly more numerous. The leproma macrophages stained more strongly with lipid stains. The color reaction to Sudan III was, however, still a yellow orange. The cells stained blue by Nile blue sulfate and did not show metachromasia. Bacilli in the lepromas were clearly stained by Fischler's method for fatty acids, and the macrophages were positively stained by the methods for phospholipids (Fig. 2). It was concluded that lipids in macrophages in murine lepromas are probably composed mainly of lipid such as phospholipids or glycolipids, or both, rather than neutral fat.

Spleen (Fig. 3). The spleens of the two to 12 week groups were studied. For each spleen, all methods listed above were performed.

By two weeks after inoculation, only one of four animals showed a few nodules. The nodules contained a limited number of bacilli and were only weakly positive to Sudan III and fett rot stains. All other methods failed to show positive results.

By four weeks, two of four animals showed a few small nodules which were weakly stained yellow-orange by Sudan III and fett rot stains, but were not stained by any other method except for firmly bound lipids. At eight weeks, the results of lipid stains were almost the same as those obtained at six weeks. Lepromas were stained by Fischler's method for fatty acids and also by all methods of phospholipids. Fischler's method revealed the contours of the bacilli clearly in the macrophages (Fig. 4). Glycolipids and cholesterol determinations were negative.

At 12 weeks, the lepromas were larger and more numerous, and stained more strongly by the methods for phospholipids (Fig. 5). However, the macrophages in lepromas were still stained yellow-orange

STAIN \ WEEKS			2	4	6	8	12
FAT RED			−~+	−~+	+	+	+
SUDAN III			−~+	−~+	+ orange	+ orange	+ orange
NILE BLUE SULFATE			—	—	blue	blue	blue
FISCHLER'S METHOD FOR FATTY ACID			—	—	+ bacilli	++ bacilli	++ bacilli
OKAMOTO'S METHOD FOR CHOLESTEROL			—	—	—	—	—
OKAMOTO'S METHOD FOR GLYCOLIPID			—	—	—	—	—
PHOSPHOLIPID	OKAMOTO'S	COMMON	—	—	+	+	++
		A	—	—	+	+	++
		B	—	—	+	+	++
	BAKER'S ACID HEMATEIN		—	—	+	+	++
	KLUVER AND BARRERA'S		—	—	+	+	++
SUDAN BLACK B WITH LIPID EXTRACT. FOR FIRMLY BOUND LIPID			—	−~+	+	+	+
GRANULOMA			−~+	−~+	+	+	+++
BACILLUS			+	+	++	++	++

FIG. 3. Lipid histochemistry of splenic leproma in murine leprosy.

by Sudan III and blue by Nile blue sulfate. Cholesterols and glycolipids were not identified in any groups up to 12 weeks after the inoculation.

In hematoxylin and eosin stains, the macrophages in the lepromas had granular cytoplasm, and usually did not show large vacuoles.

DISCUSSION

Lipid composition in murine lepra cells and its origin. Although many studies on lipid and its origin have been reported in human leprosy ([1, 3–15, 20, 24, 25, 29]), only a few investigators have similarly studied the lesions of murine leprosy ([6, 27, 28]).

As noted in Figure 6, Ueda ([27, 28]) reported that the main lipid present was probably a lecithin-like phospholipid in both human and murine leprous lesions, and neutral fat and cholesterol were present in only a small amount. Neither of the latter were identified in the present study. The discrepancy between the histochemical results for neutral fat and cholesterol between Ueda's work and this study probably depends on the state and location of the leprous lesions. Ueda used subcutaneous lepromas from eight mice which were sacrificed between 108 and 324 days after inoculation, whereas our study concerned earlier stages of splenic and peritoneal lesions between 14 and 112 days. We avoided the use of subcutaneous lepromas since skin normally has a large amount of neutral fat in the subcutis. Fite ([4]) also pointed out that the earliest bacilli-containing cells were usually free from cholesterol. Ueda ([26, 28]) and Harada ([10]) stressed that reactions to various histochemical methods for lipids are altered as lepromas age and macrophages degenerate. It is also well known in human lepromatous leprosy that macrophages in early

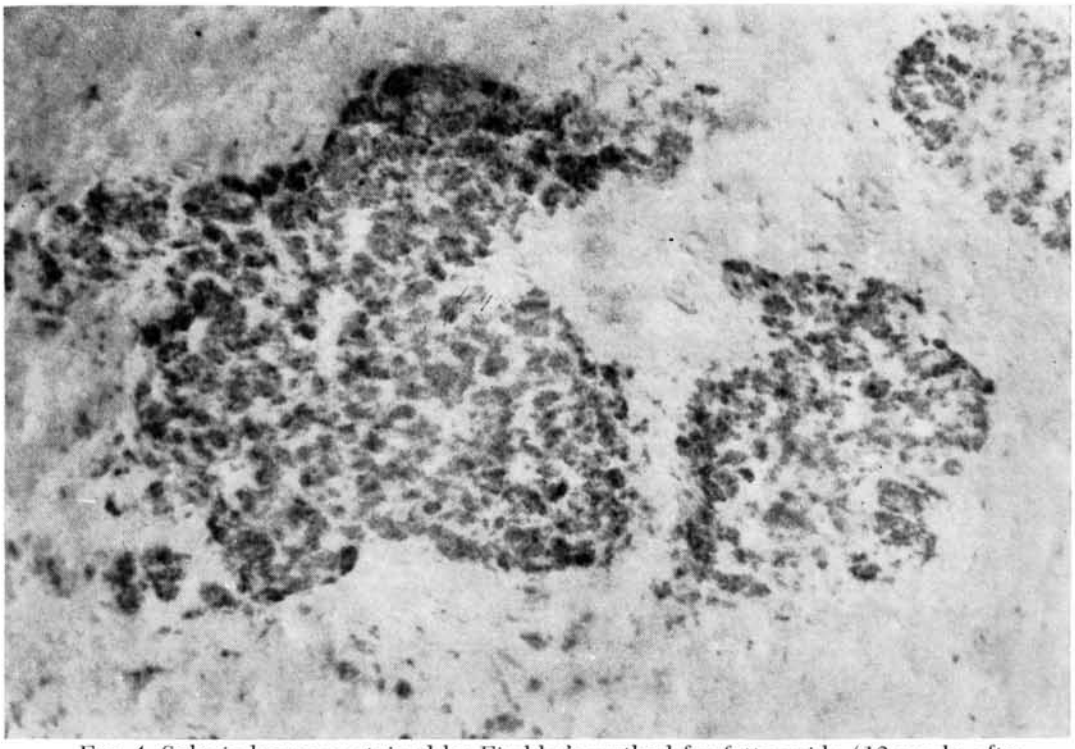

FIG. 4. Splenic lepromas stained by Fischler's method for fatty acids (12 weeks after inoculation). Original magnification X35.

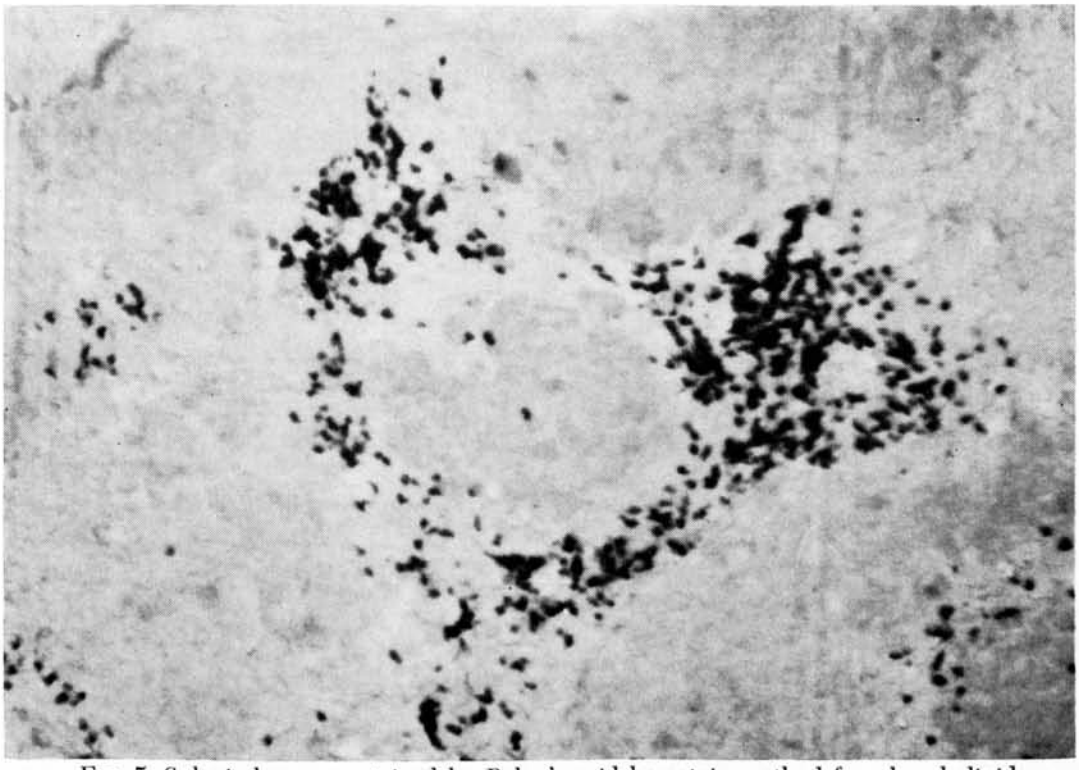

FIG. 5. Splenic lepromas stained by Baker's acid hematein method for phospholipids (12 weeks after inoculation). Original magnification X35.

		LIPIDS	NEUTRAL FAT	FATTY ACID	CHOLESTEROL		GLYCO-LIPID	PHOSPHO-LIPID	FIRMLY BOUND LIPID
					FREE	ESTER			
MURINE LEPROSY		Ueda, M.	±	−	−	±	−	+	
		Sakurai, I.	−	++ bacilli	−	−	−	++	+
HUMAN LEPROMA-LEPROSY		Ueda, M.	±	−	−	−~+	−	−~+++	
		Sakurai, I.	−~+	+++ bacilli	−	−	−	+++	−~+

FIG. 6. Comparative histochemistry of lipids in human and murine leprosy.

stages are weakly positive to lipid stains, and negative to stains for neutral fat and cholesterol. In advanced stages, the cells in the human disease are strongly stained, become positive to stains for neutral fat, and tend to present larger vacuoles which are not usually seen in murine leprous lesions ([18, 22, 24, 26, 28, 29, 30]).

In other hands Fischler's method for fatty acids is capable of staining bacilli themselves dark blue and even in early stages the macrophages in murine lepromas have been stained by various methods for phospholipids. Campo-Aasen and Convit ([2]) attempted to stain bacterial smears obtained from murine lepromas by a modified Baker's technic. They reported that murine leprosy bacilli were stained dark blue and remained positive even after pyridine-extraction, whereas human leprosy bacilli lost the blue color reaction on pyridine extraction. The Baker's technic, modified by Campo-Aasen and Convit for bacterial smears, was attempted also in the present study ([20]). Both murine and human leprosy bacilli lost the blue color reaction on pyridine extraction at 60°C for one hour, although both bacilli were positively stained by Baker's technic. Questions still remain but it is evident that a certain substance or substances stainable with Baker's method are present in the cell walls of both mycobacteria. Our results support the theory proposed by Ueda ([26-28]) and Harada ([10]) that phospholipids and fatty acids in macrophages in leprous tissues probably originate from phagocytosed bacilli. The probable reason that neutral fat and cholesterol were not identified by histochemical studies in this work is that the tissues employed were from relatively early stages of murine leprosy and the materials used were splenic and peritoneal lepromas. Certain investigators believe that neutral fat and cholesterol shown histochemically in advanced stages are probably due to cell degeneration (lipophanerosis) ([10, 24, 26-28]).

Comparison with human leprosy. Some differences in histochemical characteristics are evident on comparing the results of lipid histochemistry in murine leprosy with those in human leprosy. The intensity of color reaction in stains for phospholipids are more prominent in human leprosy than in murine lesions ([19, 28]). Reaction to firmly bound lipids is nearly always positive in murine leprosy, whereas it is frequently negative in human lepra cells except for B.663 treated leprosy ([18]). The lipids, firmly bound with protein or polysaccharides, which can not be removed from tissues or bacilli by extraction with ordinary fat solvents such as alcohols, chloroform, pyridine, acetone or their mixtures, seem to be present in greater amount in cells of murine lepromas than in human lepra cells. A summary of differences in histochemical characteristics of murine and human lepromas is shown in Figure 6.

Some morphologic differences between murine and human lepra cells have been demonstrated by electron microscopic studies, as reported by Yamamoto *et al.* ([29, 30]), Sato ([22]) and Toda *et al.* ([25]). In addition to lack of large globule formation in murine lepra cells, opaque droplets

and electron dense membranes in the foamy stuctures seen in human lepra cells are not distinct in murine cells, though both types of cells seem to contain lipids stainable with Sudan dyes ([29]). Neutral fat is sometimes demonstrated by lipid histochemistry in human lepra cells, probably because they are in advanced stages with degeneration (lipophanerosis) of macrophages contributing to large fat globule formation ([7, 10, 19, 24, 28]).

Problems in the field of lipid histochemistry. Even though neutral fat and cholesterol were not identified histochemically in this study, their existence in macrophages can not be denied, since histochemical methods may not be sensitive enough to identify a small quantity of lipids by color reaction within cells, as Okamoto *et al.* ([15]) pointed out. Toda and Nishiura ([25]) reported from electron-microscopic studies that the main lipid component in murine lepra cells may be neutral fat. Biochemical analyses of lipids in leprosy performed in our laboratory by a thin-layer chromatography (TLC) technic ([21]) have indicated the presence of almost all major lipid fractions such as phospho- and glycolipid fraction, free fatty acids, triglycerides, free and esterified cholesterols and methyl esters of fatty acids in purified murine leprosy bacilli as well as in murine lepromas (Fig. 7). Quantitative analysis of each lipid class are in progress. From an observation of the staining intensity and size of concentration areas on thin-layer chromatography plates, it appears that there may be a great deal of phospho- and glycolipid fractions and free fatty acids in both purified murine leprosy bacilli and lepromas, and more free cholesterol in cutaneous lepromas than in purified bacilli, despite far less cholesterol in normal skin controls (Fig. 4). The murine lepromas used for chromatographic studies have

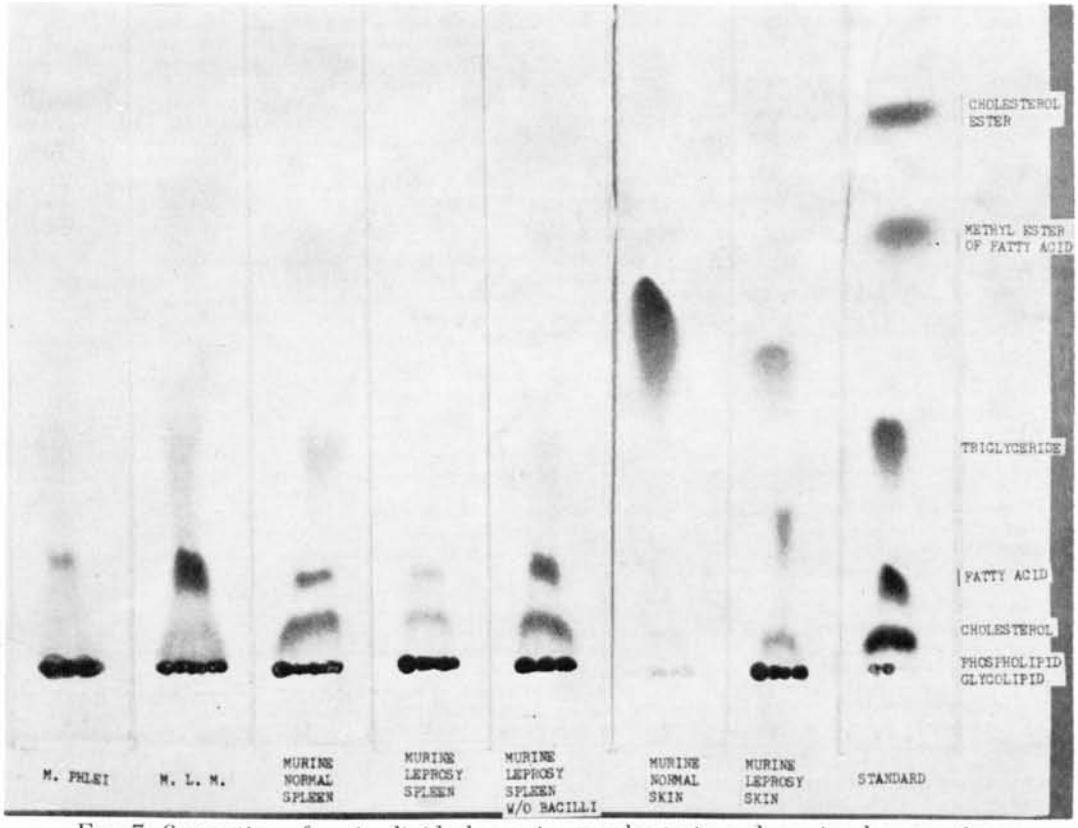

FIG. 7. Separation of major lipid classes in mycobacteria and murine leprous tissues by a thin-layer chromotography. *Thin-layer plate*: silica gel, 250 μ thick (Analtech). *Solvent*: petroleum ether-diethyl ether-acetic acid (90:10:1). *Detection*: 100% sulfuric acid with charring. Samples applied 1 $\pm$ 0.2 mg of total lipids in chloroform.

been from advanced stages of infection of five to eight months after inoculation of bacilli. These preliminary chromatographic analyses ([21]) also support the theory advocated by Ueda ([28]) and Harada ([10]) concerning origin of stored lipids in leprosy. They suggested that cholesterol and neutral fat may be deposited in lepra cells as a result of lipophanerosis, whereas lipoid substances containing phospholipids, and fatty acids probably originated from phagocytosed bacilli.

SUMMARY

A battery of histochemical methods were employed to identify lipid components in lepra cells from murine peritoneal and splenic lesions, produced by intraperitoneal inoculation of crude suspension of *M. lepraemurium*. The animals were sacrificed from two to 16 weeks after inoculation.

The major lipid components of murine lepra cells are thought to be phospholipids, fatty acids and firmly bound lipids.

Neither neutral fat nor cholesterols were demonstrated.

Fatty acids and phospholipids in murine lepra cells probably originate from phagocytosed murine leprosy bacilli.

Differences in lipid components, identified histochemically, between murine and human lepra cells include stronger staining by Baker's technic for phospholipids in human than in murine lepra cells, and less firmly bound lipids in human than in murine lepra cells.

It is pointed out that histochemical methods for lipids are not sensitive enough for identification of a small amount of lipids. More accurate and sensitive quantitative and qualitative biochemical studies are in progress.

RESUMEN

Se empleó una serie de métodos histoquímicos para identificar los componentes lipídicos de las celulas de lepra que se encuentran en lesiones peritoneales y esplénicas de lepra murina, producida por inoculación intraperitoneal de suspensiones gruesas de *M. lepraemurium*. Los animales se sacrificaron entre dos y 16 semanas después de la inoculación.

Se considera que los principales componentes lipídicos de las células de lepra murina son fosfolípidos, ácidos grasos y lípidos fuertemente enlazados.

No se evidenciaron ni grasa neutra ni colesterol.

Los ácidos grasos y los fosfolípidos de las células de lepra murina probablemente se originan de los bacilos de lepra murina fagocitados.

Se encuentra que la diferencias entre los compuestos lipídicos de las células de lepra murina y las de lepra humana, utilizando métodos histoquímicos de identificación, consisten en tinción más intensa por el método de Baker para fosfolípidos de las células humanas que de las células de lepra murina y que en las células de lepra humana se encuentran lípidos menos fuertemente enlazados que en las células de lepra murina.

Se destaca que los métodos histoquímicos para lípidos no son lo suficientemente sensibles como para identificar pequeñas cantidades de lípidos. Están en desarrollo estudios en los cuales se están utilizando métodos bioquímicos, que son más exactos y más sensibles, tanto cuantitativa como cualitativamente.

RÉSUMÉ

Une batterie de méthodes histochimiques a été utilisée en vue d'identifier les constituants lipidiques dans les cellules lépreuses provenant de lésions péritonéales et spléniques chez le rat, produites par l'inoculation intrapéritonéale d'une suspension brute de *M. lepraemurium*. Les animaux ont été sacrifiés à un intervalle variant de 2 à 16 semaines après l'inoculation.

On considère que les constituants lipidiques principaux des cellules lépreuses de lèpre murine sont des phospholipides, des acides gras et des lipides fortement liés.

On n'a démontré ni graisse neutre, ni cholestérol.

Les acides gras et les phospholipides de cellules lépreuses de la lèpre murine ont probablement leur origine dans des bacilles de la lèpre murine phagocytés.

Parmi les différences dans les constituants lipidiques, notées entre les cellules lépreuses chez le rat et chez l'homme, et identifiées par des méthodes histochimiques, on a relevé que la technique de Baker permettait de colorer plus fortement les phospholipides dans les cellules lépreuses de l'homme que dans celles du rat, et moins fortement les lipides fermement liés dans les cellules lépreuses de l'homme que dans celles du rat.

Il est à remarquer que les méthodes histochimiques pour la coloration des lipides ne sont pas assez sensibles pour identifier de petites quantités de lipides. Des études portant sur des méthodes biochimiques plus précises et plus sensibles, tant quantitatives que qualitatives, sont actuellement menées.

REFERENCES

1. Arakawa, I., Cytological studies on leprous tissue. La Lepro **27** (1958) 114-125. (In Japanese, English summary)
2. Campo-Aasen, I. and Convit, J. Indentification of the noncultivable pathogenic mycobacteria *M. leprae* and *M. lepraemurium*. Internat. J. Leprosy **36** (1968) 166-170.
3. Davison, A. R., Kooij, R. and Wainwright, J. Classification of leprosy. II. The value of fat staining in classification. Internat. J. Leprosy **28** (1960) 126-132.
4. Fite, G. L. The pathology and pathogenesis of leprosy. Ann. New York Acad. Sci. **54** (1951) 28-33.
5. Fukushi, K. and Narita, M. Histopathological study on genesis of leprous foam cell. La Lepro **29** (1960) 153-154. (In Japanese)
6. Fukushi, K., Kawazu, K., Sasaki, N., Yukawa, S., Takano, K., Narita, M. and Ishihara, K. Mobilization of the reticuloendothelial system in human and murine leprosy. La Lepro **31** (1962) 54-55. (In Japanese)
7. Ghosh, S.. Sen Gupta, P. C. and Mukerjee, N. Histochemical study of lepromatous leprosy. Bull. Calcutta Sch. Trop. Med. **10** (1962) 102-105.
8. Ghosh, S., Sen Gupta, P. C. and Mukerjee, N. Histochemical observations on tuberculoid leprosy. Bull. Calcutta Sch. Trop. Med. **1** (1963) 27-29.
9. Harada, K. Cytochemistry in leprosy bacillius. La Lepro **23** (1954) 271-278. (In Japanese, English summary)
10. Harada, K. Histochemical studies of leprosy, especially the mode of formation of lepra cells. La Lepro **24** (1955) 277-282. (In Japanese, English summary)
11. Imaeda, T. Experimental study of lepra cell by electron microscopy. La Lepro **27** (1958) 4-. (In Japanese)
12. Imaeda, T. Electron microscopy analysis of the components of lepra cells. Internat. J. Leprosy **28** (1960) 22-37.
13. Mitsuda, K. The significance of the vacuole in the Virchow lepra cells in certain organs. Tokyo Iji Shinshi 2066 (1918) 1-7. (In Japanese) *Translated to English*. Internat. J. Leprosy **4** (1936) 491-508.
14. Ogata, T. Some knowledge of leprosy pathology, especially the problems in classification. La Lepro **27** (1958) 305-312. (In Japanese)
15. Okamoto, K., Ueda, M., Maeda, T. and Mizutani, A. *Lipids, in Microscopy Histochemistry*. Chap. 4. Tokyo, Igaku-shoin, 3rd ed. 1965 pp. 331-379. (In Japanese)
16. Pearse, A. G. E. Lipids, lipoproteins and proteolipids, and Appendices 12, Chap. 12. *In: Histochemistry Theoretical and Applied*. Vol. 1. Boston, Little, Brown & Co., 3rd. ed., 1968, pp. 398-446 and 687-704.
17. Rothstein, R. The use of carbowax sections in the histochemical identification of lipids and other intracellular chemicals in amphibian and mammalian tissues. Turtox News **44** (1966) 34-37.
18. Sakurai, I. Histochemical study on hyperpigmented skin (ceroid-like pigmentation) in human leprosy treated with B.663. Fed. Proc. **28** (1969) 777.
19. Sakurai, I. and Skinsnes, O. K. Studies on lipids in leprosy. II. Histochemistry of lipids in human leprosy. Internat. J. Leprosy **38** (1970) 389-403.
20. Sakurai, I. and Skinsnes, O. K. Unpublished data.
21. Sakurai, I. and Skinsnes, O. K. Studies on lipids in leprosy. III. Chromotographic analysis of lipids in human and murine leprosy. (*Submitted for publication*)
22. Sato, S. Some problems on the fine structure of *M. leprae* and *M. lepraemurium*. La Lepro 37 (1968) 1-10. (In Japanese, English summary)
23. Skinsnes, O. K. Comparative pathogenesis of mycobacterioses. Ann. New York Acad. Sci. **154** (1968) 19-31.
24. Sugai, K. Histopathological studies on human leprosy. IV. Histochemical analysis of abnormal fats in lepromatous leprosy, especially on the fat deposition in lymph nodes. La Lepro **27** (1958) 216-227. (In Japanese, English summary).
25. Toda, E. and Nishiura, M. Differences in electron-microscopic structures between human and murine lepra cells. La Lepro **32** (1963) 166-167. (In Japanese)
26. Ueda, M. and Kusumoto, Y. Studies on lipid substances in leprous foci (I). La Lepro **17** (1948) 22. (In Japanese)
27. Ueda, M. Studies on lipid substances in

leprous foci (II). La Lepro **18** (1949) 16-17. (In Japanese)
28. Ueda, M. On the fats of various inflammatory foci, especially leprous foci of human body and rat. Trans. Soc. Path. Japan. **37** (1948) 139-141. (In Japanese)
29. Yamamoto, T., Nishiura, M., Harada, M. and Imaeda, T. Electron microscopy of ultra-thin sections of lepra cells and *Mycobacterium leprae*. Internat. J. Leprosy **26** (1958) 1-8.
30. Yamamoto, T., Nishiura, M., Harada, N., and Imaeda, T. Electron microscopy of *Mycobacterium lepraemurium* in ultrathin sections of murine leprosy lesions. Internat. J. Leprosy **26** (1958) 111-114.